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14. ABSTRACT

Each year more than one million new cases of breast cancer are diagnosed worldwide and an estimated 370,000 women die from breast cancer. Although the vast majority of fatal breast cancer cases involve metastatic spread of the primary tumor, the formation of metastases is still a poorly understood, complex process. Identifying the early molecular changes that facilitate metastasis of breast cancer will lead to new molecular targets for prevention of metastases and improved therapies. Intriguing data from the mentor's laboratory show loss of activation of the transcription factor Stat5 during breast cancer progression and that tumors without active Stat5 have higher histological grade, increased mitotic rate, and unfavorable prognosis. Furthermore, data developed in the preparation of this dissertation indicate a substantial growth-inhibitory and pro-differentiation role for Stat5 in mammary epithelial cells. Based on these and other recent observations, we postulate that loss of Stat5 activation in breast cancer represents a progression event that leads to dedifferentiation and increased risk of metastatic invasion. Therefore, a critical analysis of the role of Stat5 in human breast cancer is warranted, including systematic efforts to identify genes directly controlled by Stat5. The recent completion of the human genome sequence presents new opportunities for global identification of Stat5 target genes. Work performed in the preparation of this dissertation has established new methodology to capture, clone, sequence, and validate physiological Stat5 DNA-binding sites in a genome-wide manner. The method can also be used to determine whether Stat5 interacts with a known Stat5-responsive promoter within a given experimental context when coupled with PCR amplification of the target DNA. Using this methodology, we have demonstrated that glucocorticoids markedly alter the pattern of chromatin access for Stat5 binding in breast cancer cells. Furthermore, we also report novel methodology to specifically identify transcripts directly regulated by Stat5, based on dominant-negative, differential suppression of Stat5 regulated transcripts and large-scale gene chip analysis. As a result of this work, rapid progress in genome-wide identification of Stat5 target genes is now possible, as well as molecular mapping of the regulatory role of Stat5 in breast cancer.

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ABSTRACT

Title of dissertation: Technologies for Genome-Wide Identification of Stat5 Regulated Genes

Matthew J. LeBaron, Doctor of Philosophy, 2003

Dissertation directed by: Hallgeir Rui, M.D., Ph.D., Associate Professor, Department of Pathology

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TECHNOLOGIES FOR GENOME-WIDE IDENTIFICATION OF STAT5 REGULATED GENES

By

Matthew J. LeBaron

Dissertation submitted to the Faculty of the Department of Pathology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2003

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LIST OF ABBREVIATIONS

Ad	Adenovirus
AML	Acute Myeloid Leukemia
ALL	T- and B-cell Acute Lymphoblastic Leukemia
ATL	Adult T-cell Leukemia/lymphoma
BSA	Bovine Serum Albumin
CIS1	Cytokine-Inducible SH2 Protein 1
CML	Chronic Myeloid Leukemia
Dex	Dexamethasone
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetate
EGF	Epidermal Growth Factor
EMSA	Electrophoretic Mobility Shift Assay
EPO	Erythropoietin
ER	Estrogen Receptor
GH	Growth Hormone
GM-CSF	Granulocyte/Macrophage Colony Stimulating Factor
HTLV-1	Human T-cell Lymphotrophic Virus 1
IFN	Interferon
IGF-1	Insulin-like Growth Factor 1
IL	Interleukin
INS	Insulin
IP	Immunoprecipitation
Jak	Janus Kinase
LIF	Leukemia Inhibitory Factor
MGF	Mammary Gland Factor
MOI	Multiplicity of Infection
OSM	Oncostatin M
PBS	Phosphate Buffered Saline
PMSF	Phenylmethylsulfonyl fluoride
Prl	Prolactin
PrlR	Prolactin Receptor
RTK	Receptor Tyrosine Kinase
SAGE	Serial Analysis of Gene Expression
Ser	Serine
SH2	Src-Homology 2
SCID	Severe Combined Immunodeficiency
SOCS	Suppressor of Cytokine Signaling
Stat	Signal Transducer and Activators of Transcription
TEMED	Tetramethylethylene diamine
TPO	Thrombopoietin
Tyr	Tyrosine

BACKGROUND

Transcription factors act at the interface between the genome and the proteome. While many transcription factors are constitutively active, the activity of others is strictly regulated by extracellular factors. Examples of factors that control transcription factor activity are membrane-permeable steroids, peptide hormones, paracrine factors, or direct cell-to-cell, contact-mediated signal transduction. Extracellular-regulated transcription factors are often referred to as latent transcription factors.

Among transcription factors, latent transcription factors are particularly important determinants of cell function, as they serve both as sensors of environmental input and executors of genetic programs. While much has been learned about the extracellular regulation of latent transcription factors in the past several decades, until the recent completion of the human genome, it has been impossible to determine the genome-wide programs induced upon activation of latent transcription factors. Complicating matters, activation of the same latent transcription factor in different cell types leads to genetic programs that are cell- and context-dependent, since the repertoire of target genes available vary with chromatin structure and the presence of positive or negative cofactors.

This dissertation describes technologies for genome-wide identification of target genes for the latent transcription factor Stat5 and establishes Stat5 as a growth-inhibitory, pro-differentiation factor in mammary epithelial cells. There are two closely homologous Stat5a and Stat5b genes that belong to the family of Signal Transducers and Activators of Transcription (Stat), which include five additional genes, Stat1, Stat2, Stat3, Stat4, and Stat6. Stat5a and Stat5b are more than 94% homologous and, consistent with this, have

very similar activities, although subtle differences exist (Grimley, Dong, and Rui 1999). Stats are unique among latent transcription factors in that they are activated by tyrosine phosphorylation (Darnell, Kerr, and Stark 1994; Schindler and Darnell 1995). In addition to describing and documenting technologies to identify Stat5 target genes, this dissertation reports progress on functional analysis of the Jak2-Stat5 pathway in mouse mammary epithelial cells, as well as the use of Stat5-Chromatin Immunoprecipitation to demonstrate glucocorticoid regulation of chromatin structure in breast cancer cells.

The central reason for the author's interest in the transcription factor Stat5 is the novel observations in the mentor's laboratory that Stat5 serves as a suppressor of early metastatic progression of human breast cancer. Part of this concept stemmed from observations of a growth-suppressive role of the Jak2-Stat5 pathway that the author has helped establish in mouse mammary epithelial cells. As one line of the laboratory's research to mechanistically explain the role of Stat5 in breast cancer metastasis, the author set out to develop methodology for genome-wide identification of Stat5 target genes in breast cancer.

Two complementary technologies are presented. One is derived from chromatin-immunoprecipitation based assays (Agarwal, Avni, and Rao 2000; Ren *et al.* 2000) and involves rapid cloning and sequencing of Stat5-bound genomic DNA. The other technology, which the author and Dr. Hallgeir Rui have termed dominant-negative differential suppression of transcription, combines large-scale gene expression analysis with use of adenoviral gene delivery of dominant-negative Stat5 to identify transcripts under direct control of Stat5. Before describing the research of this dissertation, the

author will present a background on the biology of normal and malignant breast and the function and regulation of Stat transcription factors.

Biology of the Breast

Hormonal Influences

The development and maturation of the mammary gland is a highly regulated and complex process – mediated by the contributions and interactions of numerous factors. Of specific interest in this dissertation is the highly similar nature of mammary gland development and breast cancer behavior. In fact, many of the critical contributors for mammary gland development have also been identified in oncogenesis of the breast, such as invasion, re-initiation of cell proliferation, resistance to apoptosis, and angiogenesis (Wiseman and Werb 2002). Additionally, the development of the mammary gland has been long recognized as influenced by both systemic hormones and stromal component contributions (Sakakura 1987, 1991).

The physiology of the breast is notable in that it is a dynamic structure in reproductive age females, while most other organs tend to only increase in size after embryogenesis (Wiseman and Werb 2002). Moreover, these structural changes in the composition and organization of the breast are not only limited to development, puberty, and pregnancy. Lobuloalveolar development (consisting of epithelial sprouts and alveoli) are seen in the initial, proliferative phase of the menstrual cycle as a result of rising steroid (estrogen and glucocorticoids) and peptide hormone levels (prolactin, insulin, insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), and growth hormone (GH)) (Longacre and Bartow 1986). Breast ductal dilation and partial

differentiation of alveolar cells is seen in response to increased progesterone levels in the luteal phase of the menstrual cycle (Lydon *et al.* 1995). The down-regulation of steroid hormones and up-regulation of prolactin secretion cause modest secretory activity in the late menstrual cycle (Fanager and Ree 1974). However, involution or apoptosis of the ductal and alveolar epithelial cells and regression of the stromal portion of the mammary gland is present at menstruation as a result of the loss of peptide and steroid growth factors listed above (Longacre and Bartow 1986).

As described, the breast is a highly regulated and responsive organ, but the ultimate fate of the mammary gland is realized only by pregnancy and lactation (Wiseman and Werb 2002), mediated by the complex interactions of ovarian, pituitary, adrenal, and placental hormones (Schaber 1998). These hormones induce mammary epithelial cell proliferation, terminal differentiation, and secretory (milk-producing) activity, as well as the dedifferentiation of large fat cells into tiny preadipocytes (Wiseman and Werb 2002). Increased steroid hormones, specifically estrogen, in the first trimester of pregnancy is required for outgrowth in mammary gland maturation (Korach 1994), whereas progesterone has been shown to be involved in alveolar proliferation (Lydon *et al.* 1995). It should be noted, however, that prolactin is required for the terminal differentiation of mammary epithelial cells (Topper and Freeman 1980) and is also required for estrogen and progesterone's activities (Dickson and Lippman 1995). Furthermore, estrogen and progesterone increase circulating levels of prolactin by inhibiting the hypothalamic release of dopamine (a prolactin-inhibiting neurotransmitter) (Reyniak 1979). While the breast is nearly fully developed towards the end of gestation, lactogenesis is inhibited by the elevated circulating levels of estrogen and progesterone

(Hennighausen and Robinson 1998; Schaber 1998). Even with prolactin levels elevated 10-fold over the prepregnant state (Reyniak 1979) terminal and secretory differentiation is inhibited until parturition and the accompanying drop in sex steroid levels (Schaber 1998).

Suckling maintains the elevated prolactin levels, which in turn keeps the mammary epithelium terminally differentiated (Hennighausen and Robinson 1998). Weaning, and therefore milk stasis, cooperatively induce massive apoptosis and involution of the gland to the prepregnant state (Longacre and Bartow 1986). Amazingly, 90% of the mammary epithelial cells die during involution and are replaced by fat cells (Wiseman and Werb 2002). This brief overview of the physiology of mammary gland function illustrates the complex and highly synchronized interplay of hormonal regulators in the normal pregnant and non-pregnant breast.

Biology of Stat Transcription Factors

Structure

There are 7 known mammalian members of the Stat (Signal Transducer and Activator of Transcription) family, named Stat1 (Fu 1992), Stat2 (Fu *et al.* 1992), Stat3 (Zhong, Wen, and Darnell 1994b), Stat4 (Akira *et al.* 1994; Yamamoto *et al.* 1994; Zhong, Wen, and Darnell 1994a), Stat5a (Hou *et al.* 1995; Lin *et al.* 1996; Liu *et al.* 1995; Wakao, Gouilleux, and Groner 1994), Stat5b (Azam *et al.* 1995; Lin *et al.* 1996; Liu *et al.* 1995; Mui *et al.* 1995), and Stat6 (Hou *et al.* 1994) by the order in which they were identified. The Stats are initially latent, cytoplasmic, and monomeric in an unstimulated cell; each molecule is between 750-850 amino acids long, reviewed by

(Darnell 1997). (See figure 1 for a linear view of a generic Stat family member with the respective functional domains of the molecule, as well as an illustration of an activated Stat5 dimer associated with a DNA helix.) Sequence comparisons and structural and functional analyses of the Stat family members have led to a characterization of the molecules into well-defined and conserved regions, or modules of similarity (Darnell 1997).

The src-homology 2 (SH2) domain of Stats is unique in a transcription factor and is critical for activation of Stats by mediating the formation of dimers (Ihle 1996). Dimerization occurs through the interaction of a positionally conserved tyrosine residue (C-terminal to the SH2 domain, approximately amino acid (a.a.) 700) with the SH2 domain of an opposing Stat molecule (Greenlund *et al.* 1995; Shuai *et al.* 1994). This interaction is tightly regulated and requires the phosphorylation of the conserved tyrosine residue (Schindler *et al.* 1992). The mechanism of tyrosine phosphorylation will be discussed later. Formation of the dimer promotes and stabilizes the interaction of the Stat molecule with the DNA double helix through the DNA-binding domain (Becker, Groner, and Muller 1998; Chen *et al.* 1998).

The DNA-binding domain (Horvath, Wen, and Darnell 1995; Schindler *et al.* 1995) is approximately 200 amino acids long (~ a.a. 300-500) and recognizes a semi-palindromic consensus sequence, nine nucleotides in length, TTCNNNGAA (Leonard and O'Shea 1998; Xu, Sun, and Hoey 1996). Different Stats have different specificities to variants of this general consensus sequence, so a more general consensus sequence is $TTN_{4\text{-}6}AA$ (Grimley, Dong, and Rui 1999). The crystal structures of Stat1 and Stat3 revealed that the DNA binding domain is comprised of β-sheets connected by

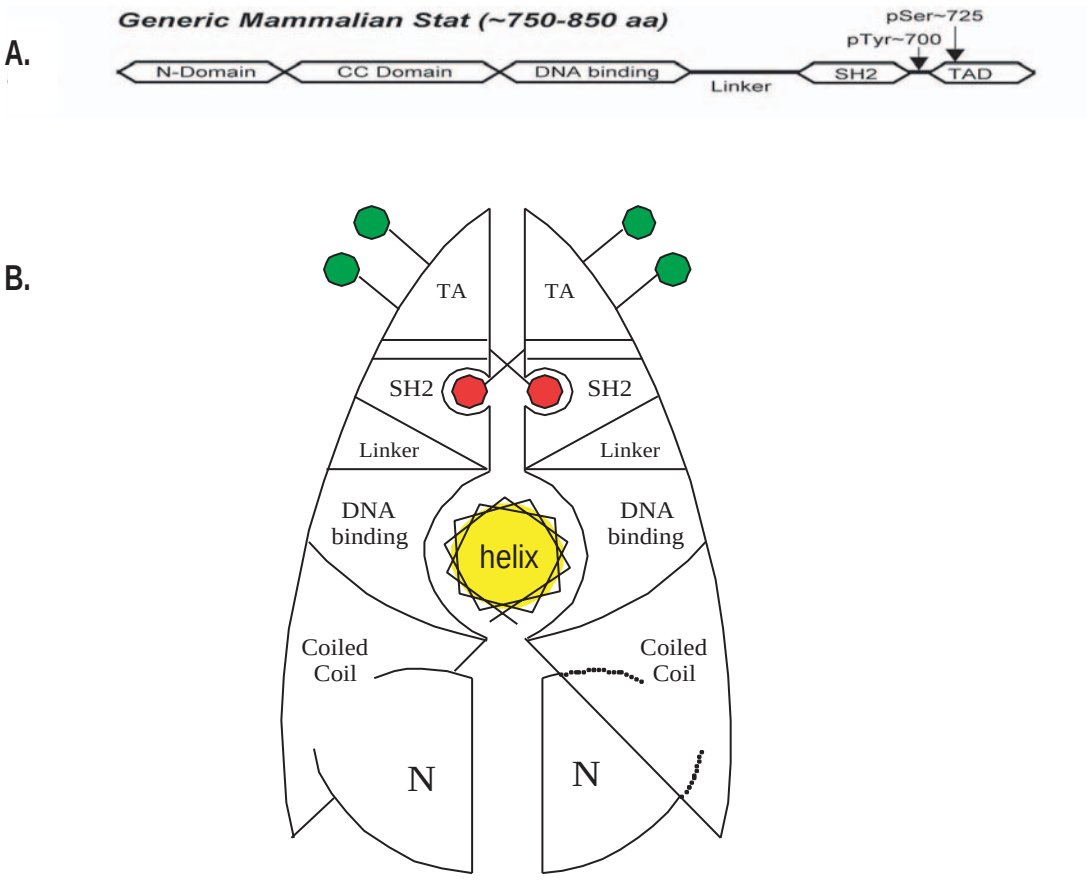


Figure 1. Generalized Stat structure and organization. A, *Linear depiction of a generic Stat molecule derived from conserved regions and elements between species.* **B,** *Functional illustration of a Stat5a tyrosine-linked, DNA-bound dimer.* Phosphotyrosine Y694 (red) forms a non-covalent interaction between molecules and stabilizes the association of the DNA-binding domain with the DNA helix (yellow). Regulatory serines S726 and S780 (green) are located in the transactivation domain. (Figure 1B modified with permission from Grimley P, Dong F, and Rui H. 1999.)

unstructured loops (Becker, Groner, and Muller 1998; Chen *et al.* 1998). Another structural domain of the Stat protein is the linker domain, interposed between the DNA-binding domain and the SH2 domain (~ a.a. 500-575). It is composed of roughly 75 amino acids that form an alpha-helical conformation (Becker, Groner, and Muller 1998; Chen *et al.* 1998) and has an unknown function (Grimley, Dong, and Rui 1999). At the extreme amino (N) -terminus of the protein is a region presumably involved in the interaction of juxtaposed Stat dimer pairs (tetramer, or dimer-dimers). This N-terminal domain may function to increase the likelihood of transcription of tandemly located Stat-responsive promoter elements (John *et al.* 1999; Verdier *et al.* 1998). Between the N-terminal domain and the DNA-binding domain is the coiled-coil domain (~ a.a. 130-300). It is composed of four helical coils of amino acids (Becker, Groner, and Muller 1998; Chen *et al.* 1998) and is thought to be important in non-covalent interactions with yet-to-be identified proteins.

At the extreme carboxy (C) -terminus is the transactivation domain. This domain is the most divergent in size and structure between Stat family members and, presumably, confers a great deal of the specificity in function (Grimley, Dong, and Rui 1999). Influences of cytoplasmic and nuclear kinases, phosphatases, and proteases may all play pivotal roles in modulating the activity of a Stat protein, the interactions of which may be mediated through the transactivation domain. Specifically, post-translational modifications, such as phosphorylation, provide an additional level of intracellular regulation to an extracellular stimulus (Eilers *et al.* 1995; Wen and Darnell 1997; Zhang, Blenis *et al.* 1995). Consistent with this regulation, work that the author has been involved in, but that is not incorporated into this dissertation, identified two proline-

directed serine phosphorylation sites within the transactivation domain of Stat5a that act as negative regulatory sites in a cooperative manner (Yamashita *et al.* 2001). Interestingly, one of the two negative sites is missing in bovine Stat5a (LeBaron, Yamashita, and Rui 2000), which may help confer increased lactation in this species (Yamashita *et al.* 2001).

Stat Function

As specified by their name, Stats are responsible for signal transduction of an extracellular signal by activating factors, as well as directly influencing gene transcription. As eluded to earlier, the transactivation domain presumably imparts the difference in function of the various Stat family members by a number of possible molecular interactions, but specificity differences exist also in the SH2 domains, DNA binding domains, and probably in the coiled-coil domains (Grimley, Dong, and Rui 1999; Levy and Darnell 2002).

The family of Stat transcription factors was initially identified and characterized in the Interferon (IFN) signaling axis of immune cells (Darnell, Kerr, and Stark 1994; Fu 1992; Fu *et al.* 1992). Further studies have identified seven mammalian Stat family members that are activated in response to a wide range of stimuli, including hormones, growth factors, cytokines, and oncoproteins (Bromberg 2000; Grimley, Dong, and Rui 1999; Leonard and O'Shea 1998). The resulting effects include cell growth, mitogenicity, differentiation, and anti-apoptotic effects, and most, if not all, cells exert their effect through at least some member of the Stat family. In an attempt to elicit the functional

differences in the individual Stat proteins, researchers have selectively disrupted each gene independently and generated knockout mice.

Stat1 deficient mice exhibit impaired response to Interferon (IFN) signaling, as well as increased susceptibility to tumors and impaired growth control (Durbin *et al.* 1996; Meraz *et al.* 1996). Not surprisingly, as Stat1 and Stat2 exhibit heterodimeric activation in response to some signals, the phenotype of the Stat2-null mouse also exhibits an impaired IFN response (Park *et al.* 2000). Elimination of Stat3 in mice results in embryonic lethality (Takeda *et al.* 1997); this phenotype is unique among the mammalian Stat family members. Subsequently, inducible removal of the *Stat3* locus by the *cre-loxP* recombination has revealed a wide-ranging effect of Stat3 function in adult tissues (Akira 2000). Specifically, defects included a failure of cell survival, impaired apoptosis, decreased immune response, and impaired wound healing. The apparent contrast in functions provides an insight into the biological complexity of Stat3. Stat4-null mice exhibit an impaired type 1 (T_H1) immune response from an inability to respond to IL-12 (Kaplan, Sun *et al.* 1996; Thierfelder *et al.* 1996). Mice without the Stat5a gene exhibit a lack of mammary gland development owing from a loss of prolactin responsiveness (Liu *et al.* 1997; Teglund *et al.* 1998), as well as a mild anemia (Socolovsky *et al.* 1999) and an epithelial defect in prostate glands (Nevalainen *et al.* 2000). As detailed in the *Stat5* section of the *Background* section of this dissertation, the highly homologous Stat5b-null mice exhibit a significantly different phenotype. These mice present with a distinct loss of sexually dimorphic growth related to impaired growth hormone signaling (Udy *et al.* 1997). Not surprisingly, the Stat5a/Stat5b double knockout mice have an additive phenotype, but also exhibit other significant changes as

well (Teglund *et al.* 1998), including severe anemia (Socolovsky *et al.* 1999), presumably due to the inability of compensation in the singly deleted mice. Stat6-null mice exhibit an impaired type 2 immune response (T_H2), presumably due to a loss of IL-4 and IL-13 responsiveness (Kaplan, Schindler *et al.* 1996; Shimoda *et al.* 1996). The specific functions and characteristics of each Stat family member are summarized in Table 1.

Stat Family Conservation

The family of Stat proteins is very well conserved in evolution. The selective advantage of multicellular organisms that could coordinate their activity provided the basis for signaling mechanisms and communication machinery (Barillas-Mury *et al.* 1999). The Stat family of transcription factors is one particular example, providing the cell a means to transmit an extracellular signal directly to gene expression. As mentioned above, the Stats are intimately involved in regulating growth, mitogenesis, differentiation, and anti-apoptotic mechanisms, all of which are crucial for the existence of a multicellular organism. For instance, the primitive metazoan *Dictyostelium* uses a Stat family protein to selectively express an extracellular matrix protein in pre-stalk cells, driving differentiation (Kawata *et al.* 1997). Similarly, *Drosophilia* Stat (D-Stat) has been shown to be involved in embryonic pattern formation and hemocyte differentiation (Hou, Melnick, and Perrimon 1996; Yan *et al.* 1996). More recently, *Anapholes gambiae*, one of the mosquitoes that carry African malaria, was identified to have a Stat protein (Ag-Stat), and, interestingly, it is activated in response to bacterial challenge in the mosquito (Barillas-Mury *et al.* 1999). Stat proteins' critical roles are exhibited by the

Table 1. Properties of Stat Family Members

	Amino Acids	*Phosphotyrosine Residue*	*Phosphoserine Residue(s)*
Stat1[a]	***750***	***701***	***727***
	Impaired response to IFN and growth control, increased susceptibility to tumors		
Stat2 [b]	***851***	***690***	***N/A***
	Impaired response to IFN		
Stat3[c]	***770***	***705***	***727***
	Embryonic lethality, impaired response to pathogens, altered cell survival in adult tissues		
Stat4[d]	***748***	***639***	***N/A***
	Loss of IL-12 responsiveness (impaired T_H1 differentiation)		
Stat5a[e]	***794***	***694***	***726,780***
	Loss of PRL responsiveness (impaired mammary gland development)		
Stat5b[f]	***786***	***699***	***731***
	Loss of GH responsiveness (loss of sexually dimorphic growth patterns)		
Stat6[g]	***847***	***641***	***N/A***
	Loss of IL-4 responsiveness (impaired T_H2 differentiation)		

[a] (Durbin *et al.* 1996; Meraz *et al.* 1996); [b] (Park *et al.* 2000); [c] (Akira 2000; Takeda *et al.* 1997); [d] (Kaplan, Sun *et al.* 1996; Thierfelder *et al.* 1996); [e] (Liu *et al.* 1997; Nevalainen *et al.* 2000; Socolovsky *et al.* 1999; Teglund *et al.* 1998); [f] (Udy *et al.* 1997); [g] (Kaplan, Schindler *et al.* 1996; Shimoda *et al.* 1996)

fact that over 200 million years of evolution exist between the common ancestor for *Drosophilia* and *A.gambiae*, yet they share greater than 40% homology of the central conserved region of the Stat (Barillas-Mury *et al.* 1999).

The seven known mammalian *Stat* genes exist in clusters on three different chromosomes, consistent with a single original *Stat* gene and subsequent duplication events (Copeland *et al.* 1995) (See Figure 2). The evolutionary lineage of the Stats is further broken into related groups based upon amino acid sequences. The A-group of Stats (presumed to be the primordial group) contains Ag-Stat, D-Stat, and the mammalian Stat5a, Stat5b, and Stat6 with an overall homology of 37.6-51.2% (Barillas-Mury *et al.* 1999). Although not as similar, the *Caenorhabditis elegans* and *Dictyostelium* Stats most resemble the A-group. B-group Stats include Stats 1-4 and have 45.2-63.4% homology at the amino acid level. Barillas-Mury, *et al* conclude it is most likely that, based upon the amino acid homology, the ancestral gene had duplicated prior to the development of vertebrates and insects (Barillas-Mury *et al.* 1999). This striking degree of homology between the Stat family members, including diverse species, indicates the substantial and conserved role for Stat proteins in mediating an intracellular response to extracellular signals.

Stat5

Stat5 was initially cloned as mammary gland factor (MGF), due to the upregulation of activity in response to prolactin in the ovine mammary gland (Wakao, Gouilleux, and Groner 1994) and shortly thereafter a report described the necessity of tyrosine phosphorylation for activation of the protein (Gouilleux *et al.* 1994). Subsequent cloning identified Stat5b as a highly related gene product and MGF was renamed Stat5a

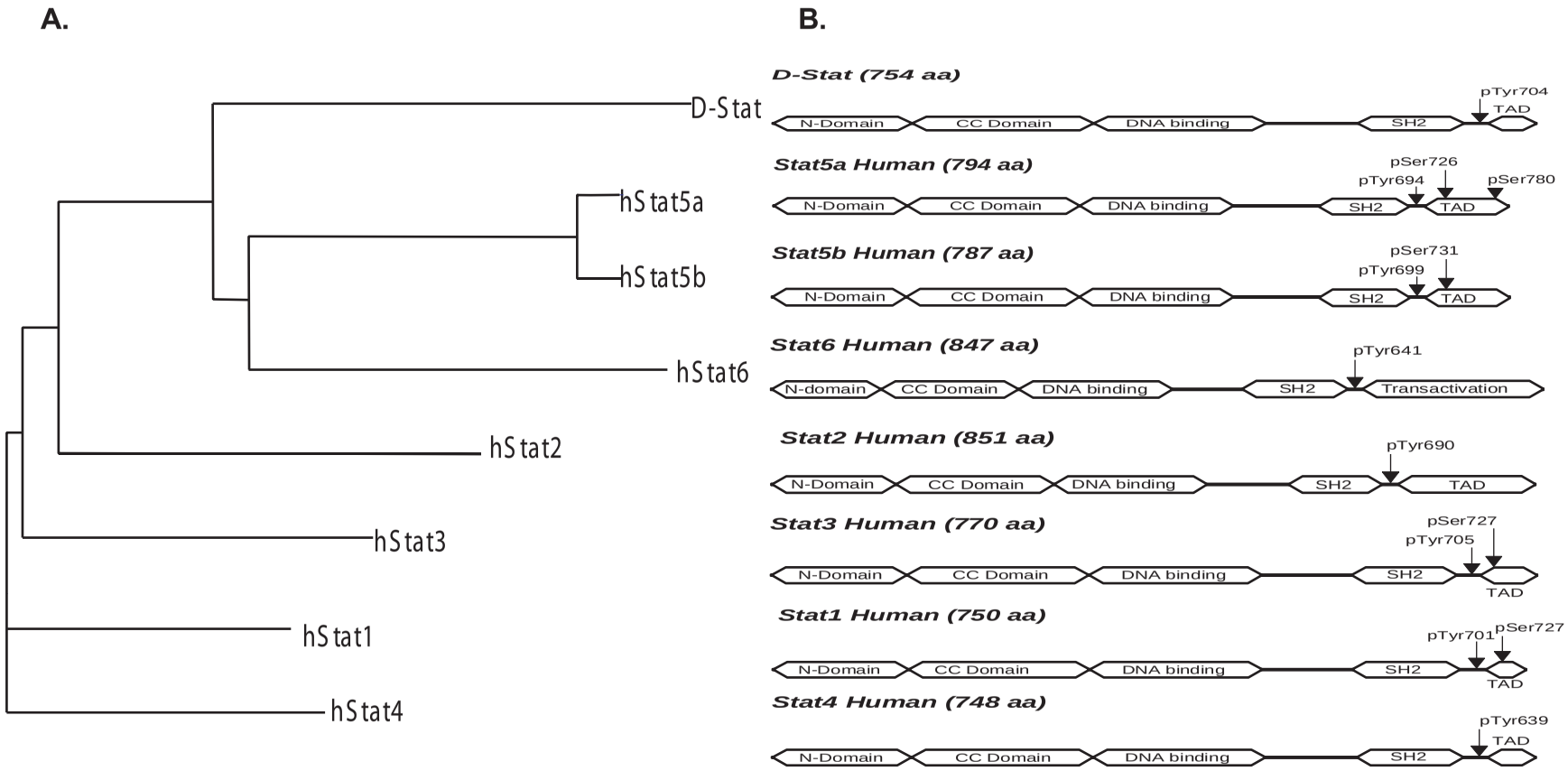


Figure 2. Stat family conservation. A. *Phylogenetic tree showing the relative comparison of the 7 human Stats and Drosophilia Stat.* **B**. *Corresponding proteins with regulatory sites and functional regions.* CC = Coiled Coil domain. TAD = Transactivation Domain.

(Liu *et al.* 1995). As previously indicated in Table 1, there is a functional difference of Stat5a and Stat5b as determined by the resultant phenotype of each knockout mouse. The two separate phenotypes may, to a large extent, be explained by tissue-specific differences in Stat5a and Stat5b gene expression, because Stat5a appears to be the major isoform expressed in prolactin target cells such as the mammary epithelial cells (Liu *et al.* 1997; Teglund *et al.* 1998), whereas Stat5b is the predominant isoform expressed in growth hormone target cells such as liver, muscle and fibroblasts (Teglund *et al.* 1998; Udy *et al.* 1997). However, minor dissimilarities in amino acid sequence of the transactivation domains and possibly of other domains of Stat5a and Stat5b may also contribute. In fact, the two proteins have greater than 95% amino acid homology throughout the molecule and a majority of the variations occur in the transactivation domain (Grimley, Dong, and Rui 1999). Of possible significance for interaction with regulatory proteins, Stat5a has two serine phosphorylation sites within its transactivation domain, whereas Stat5b only has one (Yamashita *et al.* 2001; Yamashita *et al.* 1998). Stat5a and Stat5b may also differ in their ability to be functionally activated by Src tyrosine kinases (Kazansky *et al.* 1999). Finally, one report has described minor variation in DNA binding of Stat5a and Stat5b in side-by-side comparisons and was attributed to one of the five amino acid differences between that DNA binding domains of Stat5a and Stat5b (Boucheron *et al.* 1998). Therefore, both differences in Stat5a and Stat5b gene expression and structural differences may contribute to the distinct phenotypic differences in Stat5a and Stat5b knockout mice.

A more detailed analysis of the Stat5a-null mice reveals a strong compensatory shift in Stat5b protein function to counteract the loss of the corresponding isoform. For

instance, after multiple pregnancies, Stat5b was able to partially restore lactation and became tyrosine phosphorylated in mammary glands of Stat5a-null mice (Liu *et al.* 1997; Nevalainen *et al.* 2002). Therefore, the phenotypes associated with each of the two individual Stat5 null mice are probably due to an inability to fully compensate in a cell-specific manner, either from an absolute expression deficit or from a functional difference between the molecules. In general, analysis of knockout mice must be conservatively evaluated, due to the unknown compensatory effects by homologous molecules.

An effort was made to eliminate counteracting efforts of the cell by generating double Stat5a/Stat5b knockout mice. As expected, there was an additive effect with respect to the prolactin and GH related signaling (Teglund *et al.* 1998). In addition, both sexes exhibited significant anemia from a lack of hematopoietic islands in the fetal liver (Socolovsky *et al.* 1999) and the female mice were infertile (Teglund *et al.* 1998). In summary, the proteins Stat5a and Stat5b have critical non-redundant roles in different cell-types, but also have the ability to partially compensate for the other when deleted individually. Of particular relevance for this dissertation, studies of Stat5 knockout models have established critical roles for both Stat5a and Stat5b in mammary epithelial development.

Stat5 Activation

Activation of Stat5 has been shown in a wide variety of cell types and is involved in a wide range of cellular functions (Leonard and O'Shea 1998; Levy and Darnell 2002). The importance of this pathway is underscored by the association of Jak-Stat signaling

with a wide range of membrane bound receptors for the various types of cytokines. A particularly strong association exists between Jak-Stat5 pathway activation and tetrahelical cytokines, although many other polypeptide factors may activate Jak-Stat pathways (Grimley, Dong, and Rui 1999). Tetrahelical cytokines include: cytokines IL-2 (interleukin–2), IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-13, GM-CSF (granulocyte/macrophage colony stimulating factor), OSM (oncostatin M), CNTF (ciliary neurotropic factor), GH (growth hormone), Prl (prolactin), EPO (erythropoietin), and TPO (thrombopoietin) (Leonard and O'Shea 1998). In addition, the type I interferons (IFN α/β) and type II interferon (IFN γ) are tetrahelical cytokines that also activate Jak-Stat pathways (Darnell 1997). There is marked preference of individual receptor components for interaction with particular Jak tyrosine kinases, and likewise, there is selectivity of receptor complexes for individual Stat transcription factors. For instance, prolactin preferentially activates Jak2 and Stat5, whereas IL-6 preferentially activates Jak1 and Stat3 (Leonard and O'Shea 1998). Furthermore, as described in detail later, the efficiency of the signal propagation is mediated by the near-instantaneous transmission of an extracellular signal to the nucleus by the activated Stat5 molecule itself.

In total, there are 4 Jak family members and 7 Stat family members and, of specific interest in this dissertation, the activation of Stat5 by an extracellular peptide and Jak kinase can occur by many mechanisms, and is not is not an exclusive reaction limited to one cytokine and one tyrosine kinase. In fact, as illustrated in Table 2, signals from a number of extracellular peptides are able to activate Stat5 via many Jaks (Grimley, Dong, and Rui 1999; Leonard and O'Shea 1998). In addition, Stat5 may be activated through tyrosine kinases other than Jak tyrosine kinases such as Src and Abl

Table 2. Type I Cytokine- Activators of Stat5

	Jaks	Stats
Cytokines whose receptors share γc[a]		
IL-2, IL-7, IL-9, IL-15	Jak1, Jak3	Stat5a, Stat5b, Stat3
Cytokines whose receptors share βc[a]		
IL-3, IL-5, GM-CSF	Jak2	Stat5a, Stat5b
Cytokines with homodimeric receptors[a]		
GH	Jak2	Stat5a, Stat5b, Stat3
PRL	Jak2	Stat5a, Stat5b
EPO	Jak2	Stat5a, Stat5b
TPO	Jak2	Stat5a, Stat5b

[a] [Leonard, 1998 #720][Grimley, 1999 #75]

(Kazansky *et al.* 1999), as well as the receptor tyrosine kinase for Epidermal Growth Factor (EGF) (Gallego *et al.* 2001; Taverna, Groner, and Hynes 1991).

As previously indicated, the activation of a DNA-binding Stat5 dimer is contingent upon phosphorylation of a positionally conserved tyrosine residue Tyr694 (Stat5a) or Tyr699 (Stat5b). While tyrosine phosphorylation of a Stat molecule is required for assembly of a functional Stat dimer (Darnell, Kerr, and Stark 1994; Schindler and Darnell 1995), the mechanism of activation is somewhat variable and requires thorough analysis. Additionally, although there are significant similarities in activation between the Stat family members, this dissertation will specifically detail the Jak2-Stat5 pathway, since it is regarded as a primary signaling pathway for mammary epithelial growth and terminal differentiation.

Regulation of Stat5 Activation in Mammary Epithelial Cells

Knockout mouse models have identified a critical role of PrlR-Jak2-Stat5 as a central signaling axis for alveologenesis, the development and differentiation of terminal end buds of the mammary epithelial tree into acinar structures that produce milk after parturition (Horseman *et al.* 1997; Liu *et al.* 1997; Ormandy *et al.* 1997; Teglund *et al.* 1998). This developmental step involves both a cellular expansion or growth and differentiation. Both cellular proliferation and differentiation in alveologenesis appears to be dependent on the Prl-Jak2-Stat5 axis and presumably on genes directly regulated by Stat5 (Hennighausen *et al.* 1997b; Liu *et al.* 1997). The components of this signaling pathway are illustrated in Figure 3 and the molecular details of signal transmission are

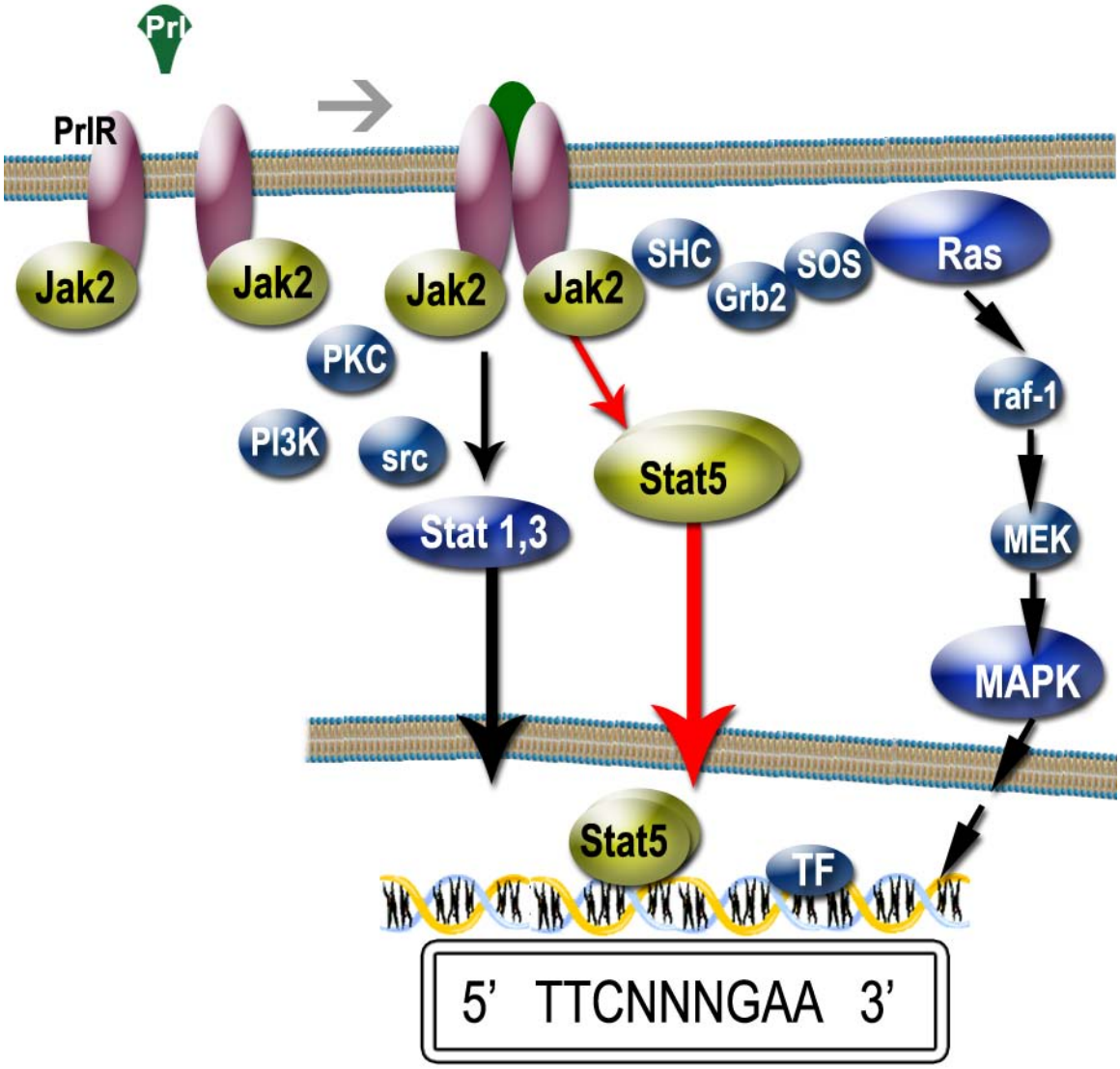


Figure 3. Overview of Prl Receptor signaling pathways. Prl causes oligomerization of prolactin receptors and activates the receptor associated Jak2 tyrosine kinase. In mammary epithelial cells, the Prl-Jak2-Stat5 pathway is the central signaling axis for mammary growth and differentiation, presumably as a result of genes regulated by the transcription factor Stat5. Stat5 binds DNA on the consensus sequence TTCNNNGAA, but has been shown to bind other sites as well.

described in the following section. To establish the role of prolactin signaling pathways in human breast cancer, a better understanding of the signaling apparatus is necessary.

Prolactin

Prolactin is a tetrahelical peptide hormone (Goffin *et al.* 1996) that is synthesized and released into the circulation by anterior pituitary lactotrophs. Human prolactin is present in the blood as monomers with a molecular weight of either 23 kilodaltons (kDa) or a glycosylated 26 kDa form (Lewis *et al.* 1985), but has also been well studied in other mammals, birds, reptiles, amphibians, and fish (Bern and Nicoll 1968; Nicoll 1980; Riddle 1963). Recent studies have also shown local production of prolactin in rodent and human breast epithelium and human breast carcinomas (Bhatavdekar *et al.* 2000; Vonderhaar 1999). Regardless of the site of production, prolactin regulates a variety of physiological processes including reproduction and lacation, growth and morphogenesis, inmmunoregulation, metabolism, behavior, and water and salt balance (Schaber 1998) and has been reviewed thoroughly (Rui and Nevalainen 2003). In fact, more than 300 distinct biological activities for prolactin have been documented (Bern and Nicoll 1968). Through genetic studies in mice and other biochemical approaches it is generally thought that a primary function of prolactin is to mediate lobuloalveolar growth and differentiation of the mammary gland for lactogenesis (Horseman *et al.* 1997; Ormandy, Binart, and Kelly 1997; Topper and Freeman 1980).

Originally described as a distinct pituitary hormone with "lactogenic" activity in 1928 (Stricker and Grueter 1928), prolactin was subsequently purified and named (Riddle, Bates, and Dykshorn 1933a, 1933b; Riddle and Braucher 1931). However, due

to the promiscuity of the prolactin receptor with closely related human growth hormones (as described below) it was not until the 1970's that human prolactin was definitively characterized as a separate hormone (Hwang, Guyda, and Friesen 1972; Lewis, Singh, and Seavey 1971).

Composition of the prolactin peptide was first determined by tryptic digestion and Edman degradation and yielded an essentially complete amino acid sequence (Shome and Parlow 1977). The determination of the primary amino acid sequence was completed by cloning of the cDNA four years later (Cooke *et al.* 1981). Comparison of the amino acid sequence with other proteins allowed the identification of highly related proteins (or families) including growth hormone and chorionic somatomammotrophin – both of which are found on human chromosome 17 (Cooke *et al.* 1981), whereas prolactin is on chromosome 6 (Owerbach *et al.* 1981). Due to the high similarity between human prolactin and growth hormone - in fact human growth hormone is able to bind and activate the human prolactin receptor - it was not until the 1970's (nearly 50 years after prolactin's discovery) that prolactin was definitively identified as a separate entity (Hwang, Guyda, and Friesen 1972; Lewis, Singh, and Seavey 1971).

Prolactin Receptor

The prolactin receptor was initially characterized as a membrane-anchored peptide receptor that had the ability to bind both human prolactin and human growth hormone (Posner *et al.* 1974), and was more thoroughly described by the cloning of the cDNA from the human breast cancer cell line T-47D (Boutin *et al.* 1989). Generally, the prolactin receptor is a typical class I cytokine receptor family member with a single-pass

transmembrane portion and the amino-terminus of the protein as the extracellular domain (Bazan 1990). Other similar tetrahelical peptide hormones that bind receptors related to prolactin receptor include: GH, EPO, CNTF, OSM, LIF, many of the interleukins, and the colony stimulating factors (Chiba, Amanuma, and Todokoro 1992; Colosi *et al.* 1993; Grimley, Dong, and Rui 1999; Leonard and O'Shea 1998; O'Neal *et al.* 1992; Quelle, Quelle, and Wojchowski 1992; Tanaka *et al.* 1992; Vigon *et al.* 1992; Yoshimura, Longmore, and Lodish 1990; Yoshimura *et al.* 1992). One of the most definitive attributes of this family of receptors is that they do not have any intrinsic kinase or catalytic function associated with the receptor, whereas receptor tyrosine kinases such as epidermal growth factor (EGF) receptor or insulin receptor (Kazlauskas *et al.* 1993; Ullrich and Schlessinger 1990; Valius, Bazenet, and Kazlauskas 1993) contain kinase domains integral to the cytoplasmic domains of the receptor (Schaber 1998). The extracellular domains of these tetrahelical cytokine receptor family share 2 pairs of disulfide linked cysteines and a Trp-Ser-X-Trp-Ser motif (Bazan 1990; Cosman 1993; Foxwell, Barrett, and Feldmann 1992; Miyajima, Hara, and Kitamura 1992), both of which have been implicated in the association of ligand and receptor (Kelly *et al.* 1993; Patthy 1990). Much of the diversity between class I cytokine receptors is present in the cytoplasmic, or carboxy-terminal, region of the receptor (Schaber 1998). In fact, several alternative cytoplasmic-domain isoforms have been identified specifically for the prolactin receptor that arise by alternative splicing in humans (Hu, Meng, and Dufau 2001; Kline, Roehrs, and Clevenger 1999), the mouse (Buck *et al.* 1992; Davis and Linzer 1989), and in the rat (Bole-Feysot *et al.* 1998).

To date, five naturally occurring forms of human prolactin receptors have been cloned, as identified and cited below. They differ mostly in their cytoplasmic domains (Buck *et al.* 1992; Ouhtit, Morel, and Kelly 1993) with exception of the ΔS1 form, which lacks approximately half of the extracellular domain and binds prolactin with lower affinity (Kline, Rycyzyn, and Clevenger 2002). The full-length, or long form of the human prolactin resceptor is comprised of 622 amino acid residues (Boutin *et al.* 1989). Additionally, there have been several shorter, or intermediate length isoforms identified for the human prolactin receptor and have been reviewed thoroughly (Clevenger *et al.* 2003; Rui and Nevalainen 2003). Specifically, one intermediate length variant of the human prolactin receptor resembles the receptor first characterized in rat Nb2 cells (Ali, Pellegrini, and Kelly 1991) and consists of 325 residues and a frameshift mutation that results in a deletion of approximately 200 amino acids from the cytoplasmic domain leaving only 13 residues, but it is still able to activate tyrosine kinase Jak2 (Kline, Roehrs, and Clevenger 1999). Another intermediate isoform for the human prolactin receptor is termed S1a, and a short variant S1b, both of which skip exon 10 and include varing lengths of the recently described exon 11 (Hu, Meng, and Dufau 2001). The S1a isoform incorporates 39 amino acids from exon 11, but appears to be less stable and perhaps of lesser biological importance than the S1b isoform, which has 3 amino acids from exon 11 (Hu, Meng, and Dufau 2001). Nonetheless, both S1a and S1b isoforms are able to suppress signal transduction to the *β-casein* promoter by the long receptor form of the human prolactin receptor in cotransfection assays (Hu, Meng, and Dufau 2001). Lastly, as eluded to earlier, another alternative transcript encodes the ΔS1 variant that lacks a significant portion of the extracellular domain of the long form of the human

prolactin receptor (Kline, Roehrs, and Clevenger 1999), while retaining the ability to mediate prolactin-induced activation of Jak2, but with an activity only 10% of the full length form (Kline, Rycyzyn, and Clevenger 2002).

The biological importance of the interaction between the human prolactin receptor splice variants or the full functional capacity of a single splice variant is yet to be determined. It important to note that prolactin receptor isoforms with variable cytoplasmic domains have been demonstrated in man, rodents, and ruminants (Bignon *et al.* 1997; Hu, Meng, and Dufau 2001) indicating a conserved role for the splice variants. Further, highly conserved splicing and tissue-specific expression patterns of prolactin receptor isoforms suggest an important biological contribution (Bole-Feysot *et al.* 1998). Since, in almost every case, the splice variants differ in the intracellular domain of the receptor the functional differences are most likely attributed to interactions with signaling proteins. For example, while complex interactions may exist, short forms may function as negative regulators of long form receptor signaling – as demonstrated in cotransfection experiments.

Janus Kinases (Jaks)

The Jaks (Janus family tyrosine kinases) are relatively large protein tyrosine kinases with an apparent molecular weight of about 120-130 kDa (~1150 amino acids) (Leonard and O'Shea 1998). Four different mammalian Jaks have been cloned and identified, namely Jak1 (Wilks *et al.* 1991), Jak2 (Wilks *et al.* 1991), Jak3 (Kawamura *et al.* 1994), and Tyk2 (Krolewski *et al.* 1990). Whereas Jak1, Jak2, and Tyk2 are more or less ubiquitously expressed, Jak3 is preferentially expressed in some differentiated

lympho-hematopoietic cell types (Gurniak and Berg 1996; Kawamura *et al.* 1994; Musso *et al.* 1995; Sharfe *et al.* 1997; Tortolani *et al.* 1995) as well as in vascular smooth muscle and endothelium (Verbsky *et al.* 1996). However, specific and unique phenotypes are associated with each of the Jak family members when selectively deleted in mice (Table 3).

A functional analysis of the specific roles Jak1 and Jak2 has been difficult to establish since mouse knockout models for either gene results in lethality. Jak1-null mice exhibit perinatal lethality, presumed to be a result of a failure of cytokine signaling in neurogenesis and hematopoietic development (Rodig *et al.* 1998). Removal of the *Jak2* locus results in an embryonic lethal mutation as a result of failed erythropoiesis (Parganas *et al.* 1998). Jak3-null mice have been established and exhibit a severe combined immunodeficiency (SCID) phenotype from a lack of signaling from the common γ chain (γc)-containing receptors (Nosaka *et al.* 1995; Thomis *et al.* 1995). Similar to the phenotypes of some of the Stat-deficient mice, Tyk2-deficient mice demonstrate increased pathogen susceptibility from impaired IFN and IL-12 responses (Karaghiosoff *et al.* 2000). The phenotypes of all *Jak*-specific null mice have been reviewed thoroughly (Levy and Darnell 2002; O'Shea 1997; Stark *et al.* 1998). While the phenotypes of Jak1 and Jak2-null mice imply a lack of redundancy in specific cytokine signal transduction, all Jak family members are involved in the propagation of extracellular signals to Stat activation.

Table 3. Properties of Jak Family Members

	Amino Acids	***Phosphotyrosine Residue***	***Active Site***
Jak1[a]	***1142***	***1022***	***991***
	Perinatal lethality from failure of cytokine signaling in neurogenesis, hematopoietic cytokine failure		
Jak2[b]	***1132***	***1007***	***976***
	Embryonic lethality from failure of erythropoiesis, immunological cytokine failure		
Jak3[c]	***1124***	***980***	***949***
	SCID from of cytokine signaling with γc receptors		
Tyk2[d]	***1187***	***1054***	***1023***
	Loss of IFN and IL-12 responsiveness (increased pathogen susceptibility)		

[a] (Rodig *et al.* 1998; Wilks *et al.* 1991); [b] (Parganas *et al.* 1998; Wilks *et al.* 1991); [c] (Kawamura *et al.* 1994; Nosaka *et al.* 1995; Thomis *et al.* 1995); [d] (Karaghiosoff *et al.* 2000; Krolewski *et al.* 1990)

Structurally, the Jaks are composed of seven domains (See Figure 4) (Leonard and O'Shea 1998; Levy and Darnell 2002). The C-terminal (JH1) domain is the catalytic region involved in the transmission of signaling via tyrosine phosphorylation. The pseudokinase (JH2) domain, located adjacent and N-terminal to the kinase domain, appears to have a negative regulatory role for the enzyme activity (Saharinen and Silvennoinen 2002; Saharinen, Takaluoma, and Silvennoinen 2000; Saharinen, Vihinen, and Silvennoinen 2003). The function of the other domains of Jaks is poorly understood. However, one consistent fact of the Jaks is the ability of the N-terminal portion of the protein to interact with cytokine receptors, although there is a relatively high degree of diversity in that portion of the molecule (Chen *et al.* 1997; Frank *et al.* 1994; Frank *et al.* 1995; Kohlhuber *et al.* 1997; Zhao *et al.* 1995). This variability is presumably the basis for the differential activation of Jaks by different cytokine receptors. The conserved domain of the membrane bound cytokine receptor mediates this interface, and involves a proline-rich region (Box 1) and an acidic region (Box 2) of the receptors (Grimley, Dong, and Rui 1999), but the determining factors of this interaction have not been well described.

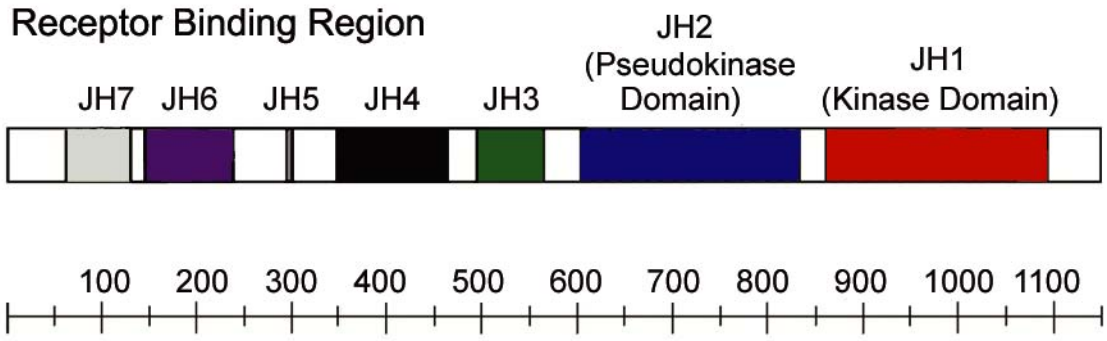


Figure 4. Generic Jak molecule structural organization. There are 7 known regions of similarity (JH1-JH7) in the 4 mammalian Jak family members. JH1 is the kinase domain and contains the active site. JH2 is similar in structure, however is not known to exhibit any kinase activity. The N-terminal domain associates with its repective receptor. Approximate amino acid location is indicated by the scale bar.

Molecular Analysis of Signal Transduction

The initial event in the propagation of a signal via the Jak2-Stat5 pathway is the binding of a ligand (prolactin for this analysis) to its cognate receptor (PrlR), instigating aggregation and dimerization of the receptor (de Vos, Ultsch, and Kossiakoff 1992; Rui, Kirken, and Farrar 1994; Windsor, Nagabhushan, Lundell, Lunn, Zauodny, Narula, and Cook 1995; Windsor, Nagabhushan, Lundell, Lunn, Zauodny, Narula, and Nakamura 1995). When brought into close proximity, the two receptor-preassociated (Leaman *et al.* 1996; Rui *et al.* 1992; Rui, Kirken, and Farrar 1994) Jak2 molecules autophosphorylate each other on key tyrosine residues within the catalytic domain and become enzymatically activated (Schlessinger and Ullrich 1992; Stahl and Yancopoulos 1993). The activated Jaks then phosphorylate tyrosine residues on the receptor (Kirken *et al.* 1993; Lebrun *et al.* 1994; Minami *et al.* 1994; Wakao, Gouilleux, and Groner 1994), allowing for recruitment of cytoplasmic and monomeric Stat5 through Stat5's SH2 domain (Pawson 1995; Schlessinger 1994). The close apposition of Stat5 with Jak2 allows Stat5 then to become phosphorylated by Jak2 on the positionally conserved tyrosine residue (Gouilleux *et al.* 1994). Subsequently Stat5 dissociates from the receptor and forms Stat5 dimers, driven by the favorable reaction kinetics of the high-affinity SH2 domain for the partner molecule's phosphotyrosine residue (Heim *et al.* 1995; Sasse *et al.* 1997). The activated Stat5 dimer is then translocated to the nucleus (Darnell 1997) through a mechanism that is still not completely understood. Regardless, activated nuclear Stat5 binds to DNA with the consensus sequence, TTCNNNGAA, and regulates gene expression as a transcription factor, as cited earlier. (Figure 3)

Tyrosine kinases other than Jaks can also activate Stats, including the direct activation of Stat5 by a RTK (receptor tyrosine kinase), such as epidermal growth factor receptor (EGF R) (Gallego *et al.* 2001; Taverna, Groner, and Hynes 1991) and platelet derived growth factor receptor (PDGF R) (Valgeirsdottir *et al.* 1998), and non-RTKs such as Src and Abl (Kazansky *et al.* 1999). Furthermore, although beyond the scope of this dissertation, numerous other factors influence Stat5 activity within the cell. Interactions of phosphatases, kinases, proteases, competitive binding factors, and points of currently unknown regulation all regulate and integrate the cellular efficacy of Stat5 function. Briefly, SOCS (suppressor of cytokine signaling) proteins are Stat responsive proteins that work to negatively regulate Stat5 activation, in an auto-regulatory fashion (Krebs and Hilton 2001). It is also believed that a nuclear tyrosine phosphatase is responsible for shutting off activated Stat5 and allowing it to be recycled to the cytoplasm, although the nuclear phosphatase has not yet been specifically identified (David *et al.* 1993; Ram and Waxman 1997). Additionally, specific phosphorylation of conserved serine residues may stimulate the activity of Stat1 and Stat3 (Wen, Zhong, and Darnell 1995; Zhang, Blenis *et al.* 1995), while phosphoserine residues serve as inhibitory sites for the activity of Stat5 (Benitah *et al.* 2003; Boer *et al.* 2002; Yamashita *et al.* 2001). Yet another layer of regulation of Stat5 activity is the cell-type specific nuclear environment that activated Stat5 encounters after translocation to the nucleus. Specifically, whether Stat5 specific response elements are available for binding depends on cell-specific chromatin structure, as well as on which cell-type specific cofactors are present (Garrels 1979). In total, the activation of Stat5 is a tightly regulated and

significant event important for many cellular functions and further research is necessary to fully appreciate the complete regulatory control involved in Stat5 function.

Stat Transcription Factors in Cancer

Much work has been done to characterize and quantify the activity of the various Stats in malignancy, as described and cited in the following sections. Although Stat1 has been shown to be activated in some cancers, the preponderance of evidence implicates Stat3 and Stat5 as the major Stat family members involved in the promotion of oncogenesis, and has been thoroughly reviewed (Bowman *et al.* 2000; Bromberg 2000, 2002). The influence of individual Stats on the development and progression of cancer is expected to vary considerably due to cell-specific differences in chromatin structure and target gene availability, as well as the cell-specific expression of cofactors that modulate Stat activity. However, several general categories of Stat involvement have been identified. These include: (1) Stats are selectively activated by oncogenic tyrosine kinase pathways; (2) dominant-negative mutants of Stat proteins suppress transformation induced by activated tyrosine kinase by blocking Stat dependent transcription; (3) constitutively active Stat mutants can induce some aspects of cell transformation, presumably by the induction of specific Stat responsive genes; and (4) inappropriate and unregulated activation of Stats in malignancy leads to the induction of genes related to proliferation and cell survival as reviewed in (Bowman *et al.* 2000). In general, the contribution of Stat activation and function is in many cases critical in oncogenesis and requires further study.

Hematopoietic Malignancies

Although not directly related to the work performed here, a majority of work on Stats in oncogenesis has been done in hematopoietic cancers and, as a result of number of different activators, hyperactivation of Stat5 is a common theme (Gouilleux-Gruart *et al.* 1996; Migone *et al.* 1995; Weber-Nordt *et al.* 1996; Zhang *et al.* 1996). Specifically, hyperactivation of Stat5 has been linked to chronic myeloid leukemia (CML) (Shuai *et al.* 1996; Weber-Nordt *et al.* 1996), acute myeloid leukemia (AML) (Gouilleux-Gruart *et al.* 1996), T- and B-cell acute lymphoblastic leukemia (ALL) (Gouilleux-Gruart *et al.* 1996; Weber-Nordt *et al.* 1996), multiple myeloma, and adult T-cell leukemia/lymphoma (ATL) (Migone *et al.* 1995) via human t-cell lymphotrophic virus infection (HTLV-I). Although this information is not directly transferable to breast cancer, it is important to acknowledge the relationships of the closely related Stat family members in various cancers, since many features of oncogenesis are consistent between cancer types. Furthermore, as previously mentioned, the primary Stat investigated in this dissertation, Stat5, has been shown to have a significant hematopoietic phenotype in the Stat5a/Stat5b-null mouse. The importance of Stat5 in oncogenesis in general may provide a hint to possible regulatory mechanisms in other tissues where Stat5 plays an equally important functional role.

Dysregulation of Jak/Stat signaling has been identified in clinical samples resulting in a childhood T-cell ALL (Lacronique *et al.* 1997), childhood B-cell ALL, and an atypical adult CML (Peeters *et al.* 1997). The driving force in these cases of malignancy was a naturally occurring mutant fusion protein that altered Jak-Stat signaling. Molecular analysis revealed a t(9;12) chromosomal translocation resulting in a

fusion protein that contained the oligomerization domain of the Tel protein (chromosome 12) bound to the Jak2 catalytic (JH1) domain from chromosome 9 (Lacronique *et al.* 1997). The subsequent molecule was shown to have constitutive tyrosine kinase activity and growth-factor independence-transforming capabilities in reliant cell lines (Peeters *et al.* 1997). As previously described, Jak2 is a normal upstream regulator of Stat5 activation in numerous cell types and, not surprisingly, its constitutive activation leads to an upregulation in the activity of Stat5. The fact that the translocation has been identified in both myeloid and lymphoid malignancies may indicate an involvement of multiple pathways by the chimeric protein (Peeters *et al.* 1997). However, the broad involvement of Stat5 in both myeloid and lymphoid lineages with regard to cellular proliferation, apoptosis, and differentiation would be consistent with a central role of Stat5 in hematopoietic malignancies. In a report using 3 human malignancy-derived variants of the Tel-Jak2 fusion gene retrovirally transfected into mouse bone marrow cells, all were shown to strongly activate Stat5 (Schwaller *et al.* 1998). Mice transplanted with these infected cells developed a rapidly fatal, mixed myeloproliferative and T-cell lymphoproliferative disorder, after a latency period of 2-10 weeks. In summary, dysregulation of Stat5 activity and Jak-Stat signaling has been shown to play a critical role in oncogenic transformation and propagation in hematopoietic cells.

Solid Tumors

Evidence for dysregulation of Stat activity has also been shown in several non-hematologic malignancies as well, although not as thoroughly investigated. The demonstration that Stat3 acts as an oncogene in mouse fibroblasts (Bromberg *et al.* 1999)

has helped support the view that constitutive activation of Stats, regardless of the mechanism, promotes oncogenesis by stimulating cell proliferation and inhibiting cellular apoptosis. However, the wide variety of Stat functions suggests numerous different alternatives – both pro- and anti-oncogenic. In fact, it is reasonable to assume that the same Stat molecule may have opposing effects, even within the same cell type, depending on the hormonal environment, tissue-specific context, and the pattern of intracellular cofactors. With this level of complexity and interaction between competing factors, magnified in the Stat family since all family members recognize the basic consensus sequence, it is reasonable to speculate that dysregulation of any Stat member may have conserved effects in cancer.

Documented involvement of Stats in solid tumors, both in primary cancers and tumor-derived cell lines have been shown to be involved in, and in some cases required for, cellular transformation as detailed and cited below. Indeed, a constitutively active Stat3 mutant that spontaneously dimerizes without the requirement for tyrosine phosphorylation can transform fibroblasts (Bromberg 2002). Although there is no recognized naturally occurring mutants of Stat3 that are known to function in this way, Stat3 is still classified as a *bona fide* oncogene (Bromberg *et al.* 1999). Dysregulation of Stat signaling has been shown to be oncogenic, for example, in cases of thyroid cancer associated with an aberrantly regulated RET receptor tyrosine kinase. The phosphorylation and activation of Stat3 is required for continued transformation and can be specifically induced by a constitutively activate member of the RET receptor tyrosine kinase family, MEN2A (Schuringa *et al.* 2001). Additionally, by removing an endogenous inhibitor of Stat3 activation, SOCS1, Yoshikawa, et al. showed that Stat3 is

constitutively activated and directly leads to transformation of a hepatocellular carcinoma-derived cell line (Yoshikawa *et al.* 2001). Recently, in primary tumors and cell lines derived from melanoma, investigators showed that Stat3 is activated in a majority of samples analyzed. Furthermore, the researchers established a requirement for the activation of Stat3 in the propagation of the malignant phenotype by inhibiting activation and observing an induction of apoptosis (Niu *et al.* 2002).

Yet another solid tumor that has been shown to be critically dependent on Stat activation for a transformed phenotype is prostate cancer. In primary prostate cancer specimens and prostate cancer-derived cell lines, Stat3 has been shown to be constitutively activated. With the administration of a specific antisense to Stat3, the cells underwent apoptosis, implying a specific requirement for Stat3 activation for the maintenance of the malignant behavior (Campbell *et al.* 2001; Gao *et al.* 2001). Furthermore, work that the author was involved in, but was not included in this dissertation, indicated that genes regulated by Stat5 promoted the survival of both androgen-independent and androgen-dependent prostate cancer cell lines (Ahonen *et al.* 2003).

Targeting of Stat3 has also shown to be effective in the abrogation of a malignant squamous cell carcinoma of the head and neck. Disrupting Stat3 function leads to growth inhibition and increases apoptosis in primary tumors and tumor derived cell lines (Song and Grandis 2000).

Additional primary tumors and cell lines derived from several different tumor types have also been shown to have aberrant Stat activation and function, although have not yet conclusively been shown to be dependent on Stat activation for maintenance of

the malignant phenotype as those listed above. These include ovarian cancer, lung cancer, brain tumors including gliomas, pancreatic cancer, and renal carcinoma (Bowman *et al.* 2000).

Characteristics of Breast Malignancies

Neoplasia in general is commonly attributed to the loss of control in one or more aspects of cellular growth, proliferation, differentiation, and survival; and malignancy of mammary epithelium is no exception. As previously mentioned, breast epithelial cells must integrate the effects of a wide array of divergent and/or synergistic signals to manage mammary function (Schaber 1998). In addition, due to its highly proliferative nature and high responsiveness to extracellular and autocrine/paracrine signaling (Hennighausen and Robinson 1998; Wiseman and Werb 2002), mammary epithelium is especially prone to accumulate mutations (Schaber 1998). As described by Kinzler, *et al*., cells with an increased necessity to proliferate tend to accumulate and propagate isolated mutations (Kinzler and Vogelstein 1996). These mutations will have an additive effect on, among other sites, tumor suppressor genes and DNA-repair mechanisms, both of which lead to genetic instability – a trait that characterizes transformed cells (Klein 1990; Lengauer, Kinzler, and Vogelstein 1998; Schaber 1998). These factors, along with inherent risks associated with the physiology of the breast, all influence the rate of malignancy in mammary epithelial tissue.

Relevant to this dissertation is the involvement of Stat5 in breast cancer. It is well established that Stat5 is critical for normal development and function of the mammary gland (Liu *et al.* 1997; Teglund *et al.* 1998). Work with transgenic mice as well as cell

culture has established the importance of Stat5 in cellular differentiation, cell survival, and growth control of normal breast epithelial cells (Li *et al.* 1997; Liu *et al.* 1997; Xie *et al.* 2002). As detailed in the next section, several lines of evidence also suggest a role for Stat5 in mammary gland carcinogenesis (Capuco *et al.* 2002; Humphreys and Hennighausen 1999; Miyoshi *et al.* 2001), perhaps by acting as a cell survival factor (Ren *et al.* 2002).

Mammary gland development involves the formation of ductal epithelial cells as well as lobular alveolar epithelium. Estrogen and EGF signaling are critical for the formation of the ductal epithelial cells while prolactin and progesterone are involved in the formation of the lobules (Bromberg 2000). Further experiments have shown the critical mediator of prolactin and EGF signaling to be Stat5, which must be present for mammopoiesis and lactogenesis (Bromberg 2000).

Mammary gland growth and development appears to be tied to malignant behavior, since many of the basic, underlying biological processes are related (Wiseman and Werb 2002). However, there is no definitive relationship known between organogenesis and cancer. One can speculate that the normal signaling pathways and proteins may become altered, either activated or suppressed, leading to uncontrolled growth or a lack of differentiated cells. Along these lines several Stat responsive genes have been coincidentally been identified as having significant prognostic significance in breast cancer (Bromberg 2000). Although no definitive link has yet been made, some prospective Stat5 responsive genes include: *Cyclin D1* (Wang *et al.* 1994; Weinstat-Saslow *et al.* 1995), *c-myc* (Berns *et al.* 1992; Guerin *et al.* 1988; Nass and Dickson

1998), *CDKN1A* (*p21*$^{WAF1/CIP1}$) (Bellido *et al.* 1998; Matsumura *et al.* 1997), and *BCL-*X_L (Liu *et al.* 1998; Nakopoulou *et al.* 1999; Vakkala *et al.* 1999).

Role of Prl-Jak2-Stat5 Signaling in Mammary Physiology

Prolactin has been shown to be a tumor promoter of the mammary gland in rodents by a variety of experimental approaches, including prolactin over-expressing transgenic mice (Wennbo and Tornell 2000). Likewise, a positive role for prolactin in mammary carcinogenesis was supported by reduced incidence of mammary tumors in mice lacking the *prolactin* gene (Vomachka *et al.* 2000). The role of prolactin in the etiology and progression of breast cancer in humans has been controversial, mainly due to the lack of a simple correlation between circulating prolactin levels and breast cancer incidence (Vonderhaar 1998) and that pharmacological suppression of pituitary prolactin secretion has had inconsistent impact on tumor growth (Llovera, Touraine *et al.* 2000). However, a large prospective study recently showed a positive correlation between circulating prolactin and increased risk of breast cancer in post-menopausal women (Hankinson *et al.* 1999). Furthermore, several laboratories have detected local production of prolactin in rodent and human breast epithelium and human breast carcinomas, and accumulating evidence suggests that prolactin can act as an autocrine mammary growth factor (Bhatavdekar *et al.* 2000; Vonderhaar 1999). Consistent with a local growth stimulatory role of prolactin, prolactin receptor antagonists inhibited the growth of several human breast tumor cell lines cultured in the absence of exogenous lactogenic hormones (Chen *et al.* 1999).

A large proportion of human breast tumors express prolactin receptors. Recent estimates indicate that more than 95% of human breast cancer biopsies are positive for

prolactin receptors (Reynolds *et al.* 1997) and many tumor-derived cell lines express high levels of prolactin receptors and can proliferate in response to prolactin *in vitro* (Shiu 1979). Finally, prolactin also was recently shown to stimulate motility of human breast cancer cells (Maus, Reilly, and Clevenger 1999), suggesting a role in promoting invasion and metastasis. Accumulating evidence therefore suggests a breast tumor promoting effect of prolactin also in humans; a series of recent reviews support this notion (Goffin *et al.* 1999; Llovera, Touraine *et al.* 2000; Vonderhaar 1999; Wennbo and Tornell 2000).

As previously mentioned and illustrated in Figure 3, prolactin receptors may activate several parallel intracellular signaling pathways. In addition to the Jak2-Stat5 pathway (Hennighausen *et al.* 1997b; Rui, Kirken, and Farrar 1994), prolactin may also activate the Ras-MAPK pathway (Das and Vonderhaar 1997; Erwin *et al.* 1995), the phosphatidyl-inositol 3-kinase-AKT pathway, and the phospholipase C-PKC pathway (Grimley, Dong, and Rui 1999; Llovera, Touraine *et al.* 2000). Mitogenic effects of prolactin have to some extent been attributed to the Ras-MAPK signaling pathway (Das and Vonderhaar 1997; Llovera, Pichard *et al.* 2000). It has furthermore been assumed that prolactin activation of the Jak2-Stat5 signaling pathway contributes to growth promotion of mammary epithelial cells, as evidenced by reduced numbers of epithelial cells in mammary glands of Stat5 null mice (Liu *et al.* 1997; Teglund *et al.* 1998).

In fact, as eluded to earlier, the prevailing view in the field is that Stat5 contributes directly to mammary tumor formation (Humphreys and Hennighausen 2000). Whereas a tumorigenic role of Stat5 in the mammary gland (Humphreys and Hennighausen 1999, 2000) is consistent with the established tumor-promoting role of Stat5 in hematopoietic cancer (Bromberg *et al.* 1999; Wellbrock *et al.* 1998), evidence

for such a role in breast cancer is very scant. Thus far, a positive role of Stat5 in mammary tumorigenesis is experimentally based on the observation that mammary hyperplasia and tumor development induced by a Transforming Growth Factor-α transgene was moderately delayed, but not inhibited, in mice lacking the Stat5a gene (Humphreys and Hennighausen 1999). In addition, reduction of Stat5a expression levels significantly increased apoptosis and decreased tumorigenesis in a murine mammary gland cancer model (Ren *et al.* 2002). Data generated in the compilation of this dissertation indicate a predominant growth-inhibitory role of the Jak2-Stat5 pathway. The six week delay of onset of tumorigenesis in the Stat5a-deficient mouse model may therefore be a statistical phenomenon related to the overall reduced number of mammary epithelial cells in these mice (Liu *et al.* 1997; Teglund *et al.* 1998). Importantly, a growth-inhibitory role of Stat5 is more consistent with the well-proven and generally accepted role of Stat5 in terminal differentiation of mammary epithelial cells (Liu *et al.* 1997; Teglund *et al.* 1998). Therefore, several independent lines of evidence suggest the need to further investigate the role of Stat5 function in mammary epithelial cells.

Clinical Implications of Stat5 Activation in Breast Cancer

Several studies have been undertaken in the mentor's laboratory to determine the role of Stat5 function in a number of mammary epithelial cell samples, from primary human breast cancer samples, as well as breast cancer derived cell lines. The data will be summarized to provide background and rationale for the research and method development presented in the *Results* section of this dissertation. As previously indicated, the involvement of Stat5 in breast epithelial cells depends upon its activation

status and not necessarily on the presence of the specific protein. In an effort to specifically characterize the status of Stat5 within the cell, a specific immunohistochemical method was developed with an anti-phosphotyrosine Stat5 antibody to detect only activated Stat5 *in situ*, as thoroughly described by Nevalainen *et al* (Nevalainen *et al.* 2002) and in the *Results* section of this dissertation. This led to the realization that Stat5 is active at a basal level in normal human and mouse mammary epithelium (Nevalainen *et al.* 2002).

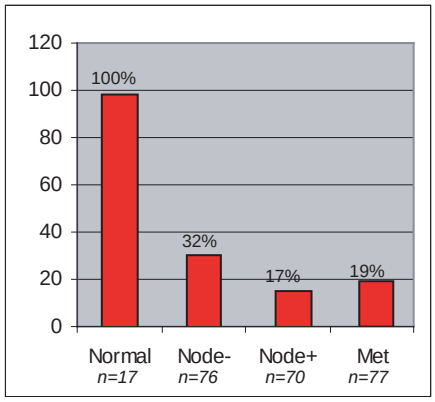
A correlation between loss of Stat5 activation and reduced differentiation in metastatic breast cancer was observed *in vitro*, as demonstrated by immunohistochemistry of active Stat5 in a series of progressively less differentiated breast epithelial lines growing in serum-supplemented medium (unpublished observations from Dr. Rui's laboratory). The loss of Stat5 signaling was also evident by immunoblotting with Stat5 antibodies of whole cell extract from the same series of progressively less differentiated breast epithelial cell lines. Specifically, in untransformed human MCF10A and untransformed mouse HC11 cells, Stat5 was expressed at high levels compared to human breast cancer cell lines T-47D, BT20, or SKBr3 (Schaber 1998). Of these cells, pretreatment with the synthetic glucocorticoid dexamethasone could to some extent restore Stat5 expression in estrogen-receptor (ER)-positive T-47D cells and to a lesser extent in MCF7 cells, but not in less differentiated, ER-negative lines BT20 and SKBr3. Furthermore, prolactin-induced tyrosine phosphorylation of Stat5 was marked in the near-normal HC11 cells, was detectable in T-47D and MCF7 breast cancer cells, but not in less differentiated BT20 and SKBr3 cells. These preliminary studies of prolactin-induced Stat5 signaling in a panel of human breast

cancer cells support the notion that Stat5 signaling is positively correlated with differentiation (Schaber 1998).

To determine whether metastatic human breast cancer in patients grows independent of basal Stat5 activation, the Stat5 activity in primary and metastatic breast cancer specimens was examined. Whereas Stat5 was continuously activated in normal human breast epithelium (100%, n=17), Stat5 was activated in only one third of primary, node-negative human breast cancer specimens. Furthermore, less than one fifth of primary, node-positive tumor samples and metastases showed active Stat5 within the epithelial component of the patient sample (Nevalainen *et al.* 2003). See Figure 5A for a graphical depiction of the Stat5 activity.

The observation that activation of Stat5 is progressively lost in primary and metastatic human breast cancer led to the investigation of a specific clinical correlation of these findings. Long-term survival analysis was evaluated using the presence or absenceof activated Stat5 in the primary tumor. In 428 patients, activated Stat5, as measured by anti-phosphotyrosine Stat5 immunohistochemistry, was determined to be a highly significant favorable prognostic marker for patient survival (Figure 5B). In addition, Stat5 activation was also positively correlated with tumor differentiation ($rho=0.4$, $p<0.001$). These observations have further fortified the basic working hypothesis that loss of Stat5 activation in breast cancer represents a progression event that leads to dedifferentiation and increased risk of metastatic invasion (Nevalainen *et al.* 2003).

A



B

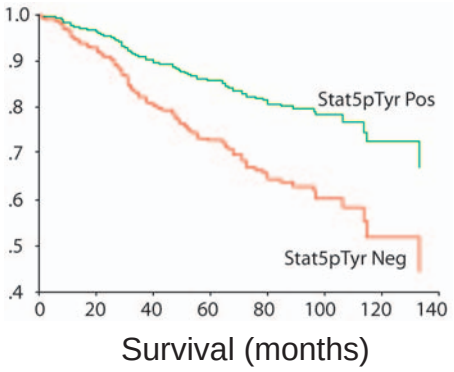


Figure 5. Stat5 activation in normal and malignant human mammary epithelium. ***A.*** *Stat5 activation is progressively lost in primary and metastatic human breast cancer.* Stat5 was continously activated in normal human breast epithelium (100%, n=17), but only 32% of primary, node-negative human breast cancer and less than 20% of node-positive tumors and metastases. ***B.*** *Stat5 is a marker of favorable prognosis in primary human breast cancer.* Specific active Stat5 immunohistochemistry provides a highly significant favorable prognositic marker for patient survival. (Modified with permission from Nevalainen MT, Xie J, Torhorst J, Bubendorf L, Haas P, Kononen J, Sauter G, and Rui H. Submitted 2003.)

Role of Stat5 in Mammary Epithelial Cell Transformation and Progression

Historically, the general notion of the field has been that prolactin promotes breast tumor formation and that Stat5 activation has a general anti-apoptotic effect in target cells (Humphreys and Hennighausen 1999; Ren *et al.* 2002). In work related to this dissertation the author participated in the development of novel and unexpected data which suggests that loss of Stat5 activation promotes increased mitosis and decreased apoptosis in non-transformed mammary epithelial cells, or a general hyperproliferative phenotype (Xie *et al.* 2002), as thoroughly described in the *Results* section. In addition, Xie, *et al.* showed a loss of breast epithelial cell contact inhibition and increased survival in an anchorage-independent environment where the activation of Stat5 was inhibited. These data, when taken together with the clinical studies, reinforce the general hypothesis that Stat5 promotes a growth-suppressive and pro-differentiation phenotype in mammary epithelial cells.

A thorough analysis of the role of Stat5 signaling in breast cells is described in the Results section of this report and provides insight into the role of active Stat5. However, these initial results have raised numerous additional questions regarding the function of Stat5 within mammary cells. Specifically, there is a need to further evaluate the role of Stat5's function as a transcription factor. While it is well known that Stat5 regulated genes play an important role in the biology of mammary epithelial cells, only a limited number of Stat5 regulated genes have been identified (Grimley, Dong, and Rui 1999) in the organism as a whole, and even fewer in a tissue specific fashion. By identifying genes directly regulated by Stat5 in mammary epithelial tissue, the present work will be

able to establish a more solid basis for the clinical and biological implications of Stat5 activation previously described. The method employed for identification of Stat5 binding sites requires several important considerations including sensitivity and specificity. The identification of Stat5 responsive genes must include novel as well as previously characterized genes (regardless of cell type) in order to ascertain the entire biological status of a particular cell. Furthermore, technology method must be developed that can identify regulatory Stat5-DNA binding sites in a genome-wide manner.

Indeed, the identification of specific binding sites for Stat5 limited to the traditional promoter regions of a gene would be largely incomplete. Several recent studies have identified binding sites for inducible transcription factors positioned in a number of unconventional positions throughout a gene locus. These include enhancer elements positioned downstream of the gene (Kim, Kelly, and Leonard 2001), intragenic loci (Frenkel *et al.* 1994), and remarkably distant sites (Li, Harju, and Peterson 1999; Muller, Gerster, and Schaffner 1988), all of which may positively or negatively regulate gene transcription. While not the traditional concept of gene transcription, where primary transcription factors bind their specific response element and recruit additional transcriptional machinery, all of the previously mentioned methods of regulation of gene expression provide specific input into the final outcome. Complementing this chromatin-based strategy to identify Stat5 target genes, effective methodology to identify Stat5-induced gene transcripts is also needed.

Chromatin Structure/Environment

For more than 40 years, scientists have recognized that proteins are encoded by the DNA sequence contained in the nucleus of a cell (Jacob and Monod 1961). Furthermore, it has been recognized for an even longer time that practically every cell within an organism contains the same genetic information, yet temporal and environmental influences alter the function of the cell or tissue individually by the presence or absence of specific proteins. Since gene expression is largely controlled at the transcriptional level (Zhang 1999), one of the most basic steps in the regulation of cellular processes is the interaction of a specific transcription factor with its respective response element.

The compaction necessary to fit over 1 meter of genomic DNA into the nucleus of a cell, which itself is only 10% of the entire cell's volume, is intrinsically restraining for DNA accessibility and functionality. In fact, the nucleus in a mammalian cell is roughly 5 μm in diameter, yet it holds 3.2 billion base pairs of DNA in addition to all associated proteins (Alberts *et al.* 1994).

The basic unit of chromatin folding is the nucleosome, which consists of 2 copies of histone proteins H2A, H2B, H3, H4 as well as a single linker histone H1 (Luger *et al.* 1997). This hockey puck like structure is wound by DNA in a left-handed superhelix approximately 2 times and is connected to the next nucleosome by 10-60 basepairs of "linker" DNA (Alberts *et al.* 1994). Each histone protein has a functional domain that promotes the association with the DNA and stabilizes the nucleosome structure as well as amino- and carboxyl-terminal tails that have the ability to be post-translationally modified. These modifications include acetylation, methylation, phosphorylation, and

ubiquitination, each of which uniquely affects the overall secondary structure of the chromatin by regulation of interactions between histone tails and DNA (Jenuwein and Allis 2001). This is believed to generally increase the overall packaging and accessibility efficiency of the genomic DNA.

Packaging considerations play an important role in the management of space within the nucleus. In fact, only 10% of the entire DNA is thought to exist in an open, fully accessible chromatin conformation (euchromatin), while 10% is packaged into a tightly condensed and inaccessible conformation (heterochormatin) (Jenuwein and Allis 2001). (See Figure 6) Heterochromatin is thought to inhibit the expression of hidden genes by physically prohibiting the interaction of general transcription factors with target DNA regions (Alberts *et al.* 1994). The remaining portion of chromatin is thought to exist in some intermediary euchromatic state, probably regulated by local factors and environmental considerations for the specific cell (Lander *et al.* 2001). This is in contrast to the historical view that chromatin was a static entity that only supported cellular machinery for various transcriptional and replicative functions. It is now clear that the mammalian genome not only contains the information in the primary sequence of DNA, but is also supplementally controlled by epigenetic mechanisms, such as those listed previously as post-translational modifications to the histones and chemical modification of DNA (Jenuwein and Allis 2001). Because there are so many different modifications that influence chromatin structure, both positively and negatively, cellular chromatin structure is likely to reflect the net effect of many different signaling pathways converging from many different stimuli.

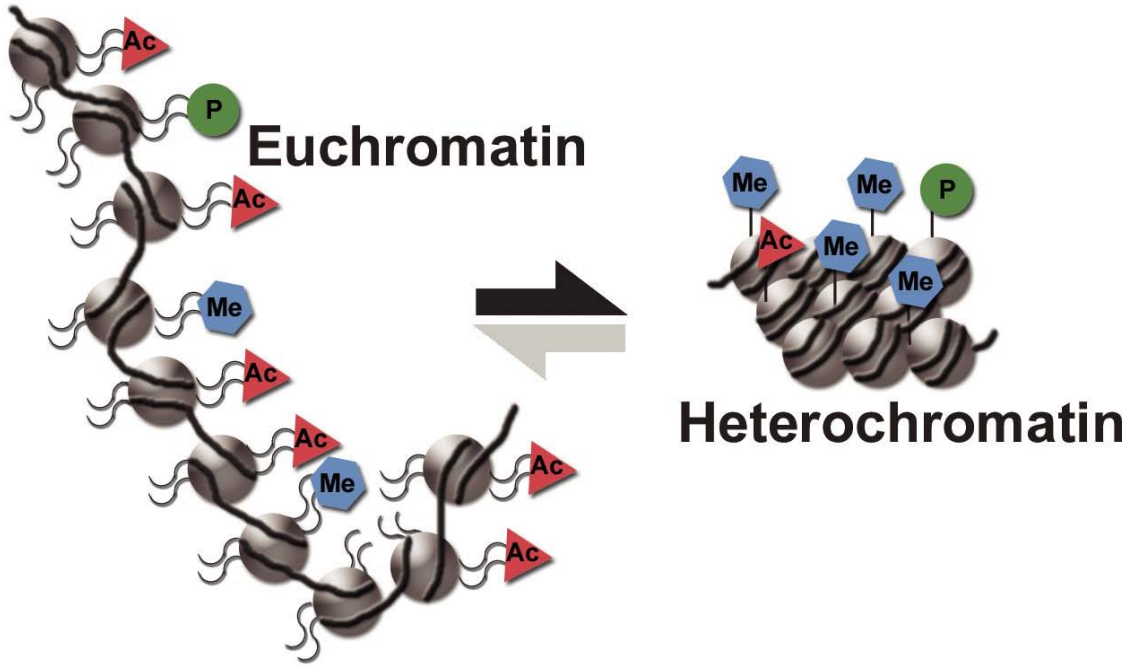


Figure 6. Chromatin structure regulates the accessibility of transcription factors to specific genomic response elements. Chromatin structure fluctuates locally between an open, accessible conformation (euchromatin) and a tightly condensed, closed confirmation (heterochromatin). Acetylation (Ac - red), phosphorylation (P - green), methylation (Me - blue), as well as other posttranslational modifications alter the structure of the chromatin and, hence, chromatin accessiblity.

Evidence for chromatin structure playing an essential role in the central workings of a cell is beginning to accumulate. Recent work has linked chromatin structure to cell cycle progression, transcription, segregation, DNA replication, DNA damage and its repair, recombination, and overall chromosome stability (Wolffe and Guschin 2000). These functions are in addition to the intuitive contributions that chromatin accessibility would play in development, stem cell lineages, and cellular differentiation. The full implications of chromatin structure are beyond the scope of this report, however, the method established and described here to identify Stat5 chromatin interaction sites provides a means to overcome many of the alternate regulatory mechanisms of chromatin structure variability and imparts an ability to identify interaction sites of a specific transcription factor with its response elements in a genome-wide manner.

Identification of Stat5 Binding Sites

From a large and yet-to-be identified pool of Stat5-regluated genes within the human genome, only a subset of genes is expected to be available for interaction with Stat5 in breast epithelial cells (Ren *et al.* 2000). As previously mentioned, cell specific availability of Stat5-chromatin interaction sites is a function of chromatin structure, DNA methylation, histone modifications, and the presence of additional cofactors. Therefore, since the goal of this approach is to identify Stat5 binding sites within the human genome, several current methodologies were evaluated for their efficacy.

Messenger RNA evaluation

The genomic code is translated into a workable platform by the transcription of messenger RNA (mRNA). This product can then be processed and transported to the cytoplasm to be translated into the protein coded in its primary sequence of nucleotides. The identification and characterization of specific mRNAs within individual cells and tissues has led to the understanding of the function of many cells and has provided invaluable insight into the organization of the human genome. Several molecular biology techniques have utilized the mRNA expression of specific genes to establish much of what is known in biology. Some of these techniques include, but are not limited to, Northern blot (Kemp, Stark, and Alwine 1977; Thomas 1980), differential display (Hedrick *et al.* 1984; Pardee and Liang 1992), SAGE (serial analysis of gene expression) (Zhang, Vogelstein *et al.* 1995), RT-PCR (reverse transcriptase - polymerase chain reaction) (DiMaio, Maniatis, and Zinn 1983; Gibbs *et al.* 1987), and large-scale gene expression analysis (gene chip) (DeRisi *et al.* 1996; Iyer *et al.* 1999; Lockhart *et al.* 1996). Each technique, while powerful, has specific drawbacks for the identification of Stat5 regulated genes in a genome wide fashion.

Of specific note is large-scale gene expression analysis, which has proved to be a very powerful tool for the investigation of transcripts and status of the cell (Alizadeh *et al.* 2000; Golub *et al.* 1999; Wan and Nordeen 2002). While the concept of comparing the relative abundance of a transcript in two different conditions is not new, the possibility of a high throughput option through microarrays is relatively new. Until recently, comparing expression levels across different tissues or cells was limited to tracking one or a few genes at a time. Using gene chip arrays, it is possible to

simultaneously monitor the activities of thousands of genes and compare the differences between two groups (Alizadeh *et al.* 2000).

In fact, global views of gene expression are often essential for obtaining comprehensive pictures of cell function. For example, it is estimated that between 0.2 to 10% of the 10,000 to 20,000 mRNA species in a typical mammalian cell are differentially expressed between cancer and normal tissues (Fey 2002). Understanding the critical relative changes among all the genes in this set would be impossible without a global approach to specifically discern the variability in gene expression. Whole-genome analyses also benefit studies where the end goal is to focus on small numbers of genes by providing an efficient tool to sort through the activities of thousands of genes, and to recognize the key ones. In addition, monitoring multiple genes in parallel allows the identification of distinct, reproducible elements of disease. Often, these gene trends are impossible to obtain from tracking changes in the expression of individual genes, which can be subtle or variable.

Gene chips also provide a reproducible framework with unparalleled sensitivity, specificity, and high-throughput when compared to other mRNA analyses. The microarray format has the ability to differentially identify splice variants (Kapranov *et al.* 2002) for the same gene, which often have an alternate, or even diametrically opposed, function. The benefits listed above, as well as the continual refinement of the techniques and tools associated with large-scale gene expression analysis, make this technology a particularly strong candidate for deciphering the functional status of breast cancer cells.

There are, however, also several limitations associated with DNA microarray technology. First, large-scale gene expression analysis is biased toward the detection of

highly abundant transcripts that increase or decrease markedly (Wan and Nordeen 2002). This is especially important to consider in the analysis of cancerous cells and tissues. For instance, key regulatory genes involved in cell cycle control fluctuate within a more narrow range than other genes associated with terminal differentiation such as milk proteins in mammary epithelial cells (Robinson *et al.* 1995). Furthermore, the same transcription factor (Stat5 in this case) may regulate the same core responsive genes either up or down, depending on the cellular and biochemical context. The down-regulation of low abundance genes is especially difficult to detect, especially when statistically compared to other highly variable transcripts (Kapranov *et al.* 2002).

Second, there are no complete genome-wide collections of human genes available for the assembly into a gene chip or chips. While the technology is rapidly improving for DNA microarray construction, the necessary information is not yet available to create a comprehensive platform for study.

Third, and most important, gene expression analysis may be inconclusive when it comes to discerning whether genes are regulated either directly or indirectly in response to a given transcription factor (Ren *et al.* 2000). For example, if Stat5 inhibits growth and stimulates differentiation of breast epithelial cells by targeting a subset of key genes, the transition from actively cycling cells to growth-arrested cells in G_0 will be associated with large secondary changes in mRNA levels of cell cycle-specific genes. That is, the initial induction of a transcript by the transcription factor of interest may lead to the generation of additional genes, which would be indecipherable on a gene chip, unless the experimental set-up is carefully designed.

Lastly, it is important to recognize some of the shortcomings of mRNA analysis in general as a tool to extrapolate the biological status of a cell or tissue. As a part of the cell's regular ability to tightly control its function, mRNA is constantly generated and destroyed (Darnell 1982; Derman *et al.* 1981). Additionally, the turnover of independent transcripts fluctuates from a number of factors creating inconsistent variability in the longevity of transcripts (Sachs 1993; Theil 1990). Therefore, the inherent instability of transcripts creates an issue of sensitivity, in particular, with respect to the previously mentioned transcripts of low abundance and high significance. It is also commonly known that mRNA levels do not necessarily equal protein, the functional component of a gene (Marzluff 1992; Wickens 1990). Numerous studies have identified complex regulatory mechanisms to inhibit the translation of mRNA into protein, essentially rendering the transcript impotent. In addition, other factors have been shown to alter the efficiency of translation for a particular transcript, influencing the ratio of message to protein (Bock *et al.* 1991).

Genome Wide Analysis

Instead of a large-scale gene expression approach to identify Stat5 target genes within the human genome, one might perform a computer analysis for Stat5 consensus binding sites (Kel *et al.* 2001). As a first draft of the human genome was published in 2001 (Lander *et al.* 2001; Venter *et al.* 2001) and completed this year (2003), the entire genome is now accessible at single nucleotide resolution and opens new possibilities for computational transcription factor research. Nonetheless, there are several limitations to computational prediction of transcription factor interaction with DNA that can only be

overcome by experimental testing. First, as with other transcription factors (Ren *et al.* 2000), it is assumed that Stat5 binding sequences located in promoters of Stat5-regulated genes, TTN_5AA, can also be found at many sites throughout the human genome where Stat5 binding is not detected (Brockman, Schroeder, and Schuler 2002). Additionally, computational prediction relies on known sequences of Stat5 binding and eliminates other possibilities for Stat5 interaction with DNA including cooperative or competitive binding with additional cofactors (Zhang 1999). Nor will computational analysis readily identify cell- and tissue-specific deviations in chromatin structure, as described earlier. The presence or absence of additional regulatory proteins and nuclear factors will all influence gene expression as well, such as histone deacetylases, histone acetylases, methylases (Jenuwein and Allis 2001), proteases and phosphatases that act directly on Stat5 (Lee *et al.* 1999).

In order to fully appreciate the difficulties of using a computer algorithm to determine Stat5 binding sites, some calculations are helpful. The generic Stat5 consensus sequence, TTNNNNNAA, which has been shown to associate with Stat5 in binding assays (Barillas-Mury *et al.* 1999; Decker, Kovarik, and Meinke 1997; Ihle 1996), is present 1.3×10^7 times within the 3.2×10^9 base pairs of DNA within the human genome. The somewhat more restrictive consensus sequence, TTCNNNGAA, would be present 8.5×10^5 times by a statistical calculation based on the random distribution of the 4 nucleotides. Further work has shown that Stat5 can associate with half sites, TTC or GAA, when associated with other transcription factors that act to stabilize the DNA binding complexes (Verdier *et al.* 1998). Statistical analysis shows that the occurrence of these half sites would be 2.2×10^8 times within the genome for either sequence. Clearly,

an exclusive computational analysis for the presence and location of Stat5 consensus sequences in the identification of binding sites would be of limited value, since most of the predicted sites would not serve as physiological binding sites in a cell-specific setting. There are far too many complicating and variable circumstances to reliably determine the location of Stat5 binding sites based purely on DNA sequence analysis. The author's collaborative work described in Chapter I provides new evidence for involvement of Stat5 in breast epithelial cell growth and differentiation, and in Chapters II-IV the author presents two new technological developments to experimentally identify Stat5 target genes in a genome-wide manner.

RESULTS

The *Results* section of this dissertation is divided into four segments or chapters, each an independent arm for establishing the role of Stat5 function in mammary cell biology. Each chapter has an *Introduction, Results, Discussion, and Materials and Methods* subsections. The four chapters are titled: I) *Role of Prl-Jak2-Stat5 Signaling in Mammary Epithelial Cell Differentiation and Growth,* II) *Development of a Method for Genome-Wide Identification of Stat5-Chromatin Interaction Sites,* III) *Strategy to Identify Stat5-Induced Transcripts Based on Dominant-Negative Differential Suppression of Transcription and RNA Gene Chip Analysis,* and IV) *Cell Differentiation-Dependent Changes in Accessibility of Specific Genomic Response Elements to Transcription Factor Stat5.* These separate and novel approaches to discern the function of Stat5 have provided significant new insight to the regulation of growth and differentiation of mammary epithelial cells.

CHAPTER I:

ROLE OF PRL-JAK2-STAT5 SIGNALING IN MAMMARY EPITHELIAL CELL DIFFERENTIATION AND GROWTH

The work presented in this section of the *Results* was originally published in the *Journal of Biological Chemistry*, volume 277(16), pages 14020-14030, by Xie J, LeBaron MJ, Nevalainen MT, and Rui H in 2002 and is cited in this dissertation (Xie *et al.* 2002). The author of this dissertation collaborated with other members of Dr. Rui's laboratory in the preparation and synthesis of this article and was a significant contributor to the work. The data provided new and unique insight into the role of Stat5 function in the mammary gland and was therefore critical in the rationale for the development of technologies described in subsequent chapters in this dissertation.

Introduction

As previously described, prolactin is a principal differentiation factor for human and mouse mammary epithelial cells and is required for milk production (Horseman *et al.* 1997). However, prolactin may also stimulate mammary epithelial cell growth and act as a mammary tumor promoter (Horseman *et al.* 1997; Wennbo *et al.* 1997). Identifying the roles of individual signaling molecules and pathways activated by prolactin in normal mammary epithelial cells is therefore needed to better understand the role of prolactin-mediated signaling in breast cancer.

Prolactin activates tyrosine kinase Jak2 (Rui, Kirken, and Farrar 1994) and transcription factor Stat5 (Wakao, Gouilleux, and Groner 1994) in target cells, including

Nb2 lymphocytes, ovarian cells, and mammary cells. Specifically, the Jak2-Stat5 pathway is expected to mediate prolactin-induced mammary epithelial cell differentiation (Hennighausen *et al.* 1997a). While genetic studies have established a critical role for Stat5 in mouse mammary gland differentiation (Liu *et al.* 1997; Teglund *et al.* 1998), corresponding genetic evidence is not yet available for Jak2 because *Jak2* null mice die *in utero* (Neubauer *et al.* 1998; Parganas *et al.* 1998), and conditional *Jak2* null mice have not been established. Although Jak2 has been regarded as the principal tyrosine kinase activated by prolactin (Rui, Kirken, and Farrar 1994), the picture has been complicated by evidence that prolactin also can activate other tyrosine kinases, including Src-family kinases (Berlanga *et al.* 1995; Clevenger and Medaglia 1994), focal adhesion kinase (Canbay *et al.* 1997), TEC kinase (Kline, Moore, and Clevenger 2001), and the ErbB-2 receptor tyrosine kinase (Yamauchi *et al.* 2000). Experimental testing of the importance of Jak2 for prolactin-induced Stat5 signaling and differentiation of mammary epithelial cells was therefore warranted. Furthermore, because the Jak2-Stat5 pathway is oncogenic in hematopoietic cells (Lacronique *et al.* 2000), it was also critical to establish the role of the Jak2-Stat5 pathway in regulating growth of normal mammary epithelial cells.

In this study, we targeted *Jak2* in an *ex vivo* model mammary epithelial cell differentiation to determine the role of Jak2 in prolactin-induced signaling in cell differentiation and growth. Two distinct targeting strategies were used to suppress Jak2 kinase activity in immortalized HC11 mouse mammary epithelial cells. First, an effective *Jak2* antisense construct was generated and stably introduced into HC11 cells. Second, a functional dominant-negative *Jak2* mutant was generated and introduced into

HC11 cells by adenoviral delivery. The following studies demonstratd that Jak2 is essential for prolactin-induced differentiation and activation of transcription factor Stat5 in normal HC11 mouse mammary epithelial cells. Importantly, suppression of Jak2-Stat5 signaling in HC11 cells was associated with a hyperproliferative phenotype characterized by increased mitotic rate, reduced apoptosis, and reduced contact inhibition. In addition, constitutive activation of Stat3 was associated with suppression of Jak2 in HC11 cells. Collectively, our data suggest that Prl-Jak2-Stat5 signaling mediates growth-suppressive and differentiation-inducing effects on normal mouse mammary epithelial cells. These observations may provide important new insight into the role of the prolactin-activated Jak2-Stat5 pathway in breast cancer.

Results

Prolactin-Induced Differentiation of HC11 Mouse Mammary Epithelial Cells Correlated With Activation of Tyrosine Kinase Jak2

Confluent, growth-arrested HC11 mouse mammary epithelial cells can be induced to differentiate *in vitro* by prolactin in medium supplemented with glucocorticoids and insulin (Ball *et al.* 1988). This differentiation process leads to formation of mammospheres, which are acinar-like structures that have been shown to express milk proteins (Blatchford *et al.* 1995). HC11 cells have been widely used as an *ex vivo* model of mammary gland epithelial cell differentiation (Humphreys and Rosen 1997; Hynes *et al.* 1990). We took advantage of this model to determine whether Jak2 was critical for activation of Stat5 and prolactin-induced differentiation.

The time-dependent differentiation of HC11 cells induced by prolactin as measured by the appearance of mammospheres is presented in Figure 7A. Mammospheres were detectable within one day of prolactin treatment, and additional mammospheres continued to form over a period of four to five days of culture, reaching a plateau at a density of approximately 15 mammospheres per cm^2. Although glucocorticoids and insulin are required supplements in the differentiation medium, mammosphere formation critically required prolactin as demonstrated by a concentration-dependent effect of prolactin ranging from 0 to 20 nM (Fig. 7B).

To determine expression and activation patterns of Jak tyrosine kinases at the initiation of differentiation treatment, HC11 cells were treated with or without prolactin for 30 min and harvested. Individual Jak kinases were immunoprecipitated from cell lysates and immunoblotted for phosphotyrosine and reprobed for Jak protein levels. These analyses showed that prolactin-treatment of HC11 cells correlated with selective activation of Jak2, and not of other members of the Jak tyrosine kinase family (Fig. 7C). Specifically, phosphotyrosine immunoblotting of immunoprecipitated Jak proteins established that only Jak2 became detectably tyrosine phosphorylated in response to prolactin. Furthermore, of the four Jak kinases, Jak2 was the only Jak family member expressed at significant levels in HC11 cells. Control experiments verified that the antibodies used for immunoprecipitation and immunoblotting of the various Jak tyrosine kinases were effective against mouse isoforms (data not shown). Therefore, prolactin-induced differentiation of HC11 mammary epithelial cells correlated with selective activation of Jak2 tyrosine kinase.

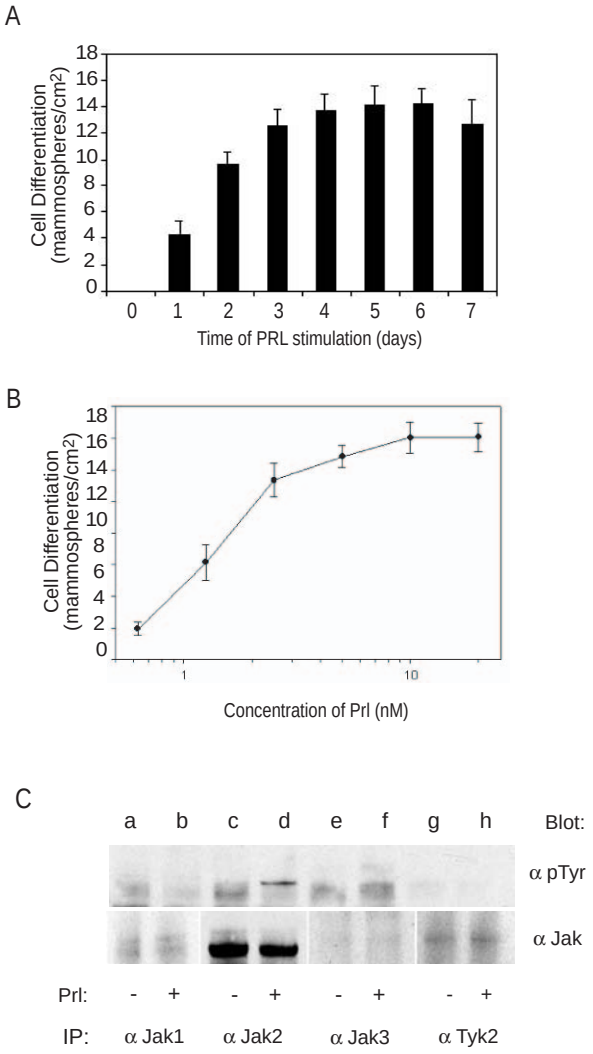


Figure. 7. Prolactin-induced differentiation of HC11 mammary epithelial cells correlated with activation of Jak2 tyrosine kinase activity, and not other Jaks. A, *Time-dependent stimulation of HC11 cell differentiation by prolactin (Prl).* HC11 cells were stimulated with prolactin (10 nM) for up to 7 days, and density of mammospheres was recorded by manual counting under phase-contrast microscopy. Data presented represent three independent experiments carried out in duplicate (error bars represent SEM). See Materials and Methods section for detailed culture conditions. **B**, *Concentration-dependent stimulation of HC11 cell differentiation by prolactin.* HC11 cells were incubated with concentrations of prolactin ranging from 0 to 20 nM for four days, and density of mammospheres were recorded. Data represent three independent experiments carried out in duplicate (error bars represent SEM). **C**, *Prolactin selectively activated tyrosine kinase Jak2, and not other Jaks, in HC11 cells.* HC11 cells at day 0 of differention treatment were exposed to prolactin (10 nM) for 30 min. Jak1, Jak2, Jak3 and Tyk2 were individually immunoprecipitated (IP) with specific antibodies, and first subjected collectively to anti-pTyr immunoblotting and subsequently reprobed individually for corresponding Jak protein levels. (Xie, LeBaron, Nevalainen, and Rui, 2002)

Jak2 Antisense Blocked Prolactin-Induced Differentiation of Stably Transfected HC11 Clones

As one strategy to suppress Jak2 function and test the importance of Jak2-mediated signaling in prolactin-induced differentiation of HC11 cells, we generated an antisense construct to inhibit Jak2 protein expression. The genetic engineering of this *Jak2* antisense construct, which targeted a region of 51 bp unique to the *Jak2* transcript, is described in detail in the *Materials and Methods* section. This region is located within the hinge region between the JH2 pseudokinase and JH1 kinase domains of *Jak2*. Functional testing of the antisense construct was first carried out using COS-7 cells and cotransfection experiments with a V5-His tagged Wt-*Jak2* (wildtype) construct. We determined that the *Jak2* antisense construct effectively blocked Wt-Jak2 expression in a dose-dependent manner under conditions where total amounts of DNA transfected were kept constant (Figure 8A). Equivalent amounts of cell lysates in the various lanes were verified by parallel blotting for β-actin. Furthermore, the control sense construct did not affect Jak2 levels in parallel experiments (data not shown).

To directly test whether Jak2 kinase activity is required for prolactin-induced mammary epithelial cell differentiation, we then generated stable HC11 clones expressing the *Jak2* antisense construct. A vector sense-control clone (HC11-Vs) was also selected for use as a second control cell line in addition to the parental line. Stable clones A and B were selected for further study from several positive clones, and showed markedly reduced Jak2 protein levels by immunoprecipitation and Western blot analysis (Figure 8B, lower panel). Correspondingly, basal and prolactin-induced Jak2 tyrosine phosphorylation was lost in clones A and B (Figure 8B, upper panel). When examined in

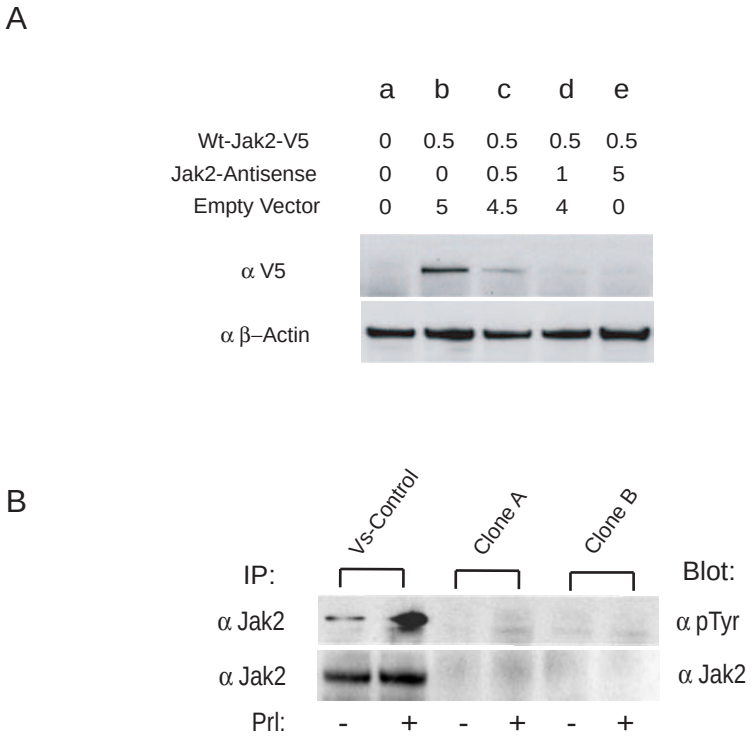


Figure 8. Antisense Jak2 blocked prolactin-induced differentiation of stably transfected HC11 clones. A, *Validation of efficiency of Jak2 antisense construct by transient cotransfection in COS-7 cells.* COS-7 cells were cotransfected with a constant amount of plasmid encoding V5-epitope-tagged *Jak2* and an increasing amount of plasmid encoding a 51 nucleotide antisense mRNA specific to *Jak2* mRNA. In each case, total levels of transfected DNA were kept constant by compensating with empty pcDNA3 vector as indicated. Levels of Jak2 protein were monitored in cell lysates by anti-V5 immunoblotting in the presence of increasing amounts of antisense-Jak2. Parallel immunoblotting for β-actin was used to verify equal loading of cell lysates. **B**, *Validation of efficiency of Jak2 antisense construct in stably transfected HC11 clones.* HC11 cells were transfected with vector sense control plasmid or *Jak2* antisense plasmid using Lipofectamine 2000. A vector sense (Vs) control clone and two antisense clones (A and B) were selected, and clones were incubated with or without prolactin (10 nM) for 30 min. Jak2 was immunoprecipitated from whole cell lysates and tested for levels of tyrosine phosphorylated Jak2 and levels of Jak2 protein by immunoblotting. (Xie, LeBaron, Nevalainen, and Rui, 2002)

differentiation assays, suppression of Jak2 levels in clones A and B was associated with complete disruption of prolactin-induced mammosphere differentiation, as illustrated by representative images of cultures on day 4 of treatment (Figure 9A). In contrast, mammosphere formation remained intact in parental HC11 and vector-sense control cells. Mammosphere formation was quantified by counting, and the data from three independent experiments were expressed as number of mammospheres per cm^2 of culture surface (Figure 9B). The inhibitory effect of Jak2 suppression on cell differentiation was equally pronounced after extended treatment with prolactin for up to 7 days (data not shown). Therefore, suppression of Jak2 levels, and subsequent Jak2 kinase activity, by stable expression of a *Jak2* antisense construct blocked prolactin-induced differentiation of HC11 cells.

Construction and Functional Testing of a Dominant-Negative Jak2 Mutant

To further test by an independent strategy whether Jak2 signaling was essential for prolactin-induced mammary differentiation, we generated a dominant-negative Jak2 protein by deletion of the C-terminal kinase domain as described in the *Materials and Methods* section. As a functional test of this construct, transient transfection assays in COS-7 cells were used to examine the ability of this kinase-deleted mutant Jak2 to specifically block prolactin-induced activation of Stat5 by Wt-Jak2. COS-7 cells were cotransfected with plasmids encoding *PrlR*, *Stat5a*, and *Jak2* forms as indicated, and stimulated with or without prolactin for 30 min (Figure 10A).

In whole cell lysates of mock-transfected, negative control cells, immunoblotting revealed no detectable endogenous Stat5, and therefore no response to prolactin

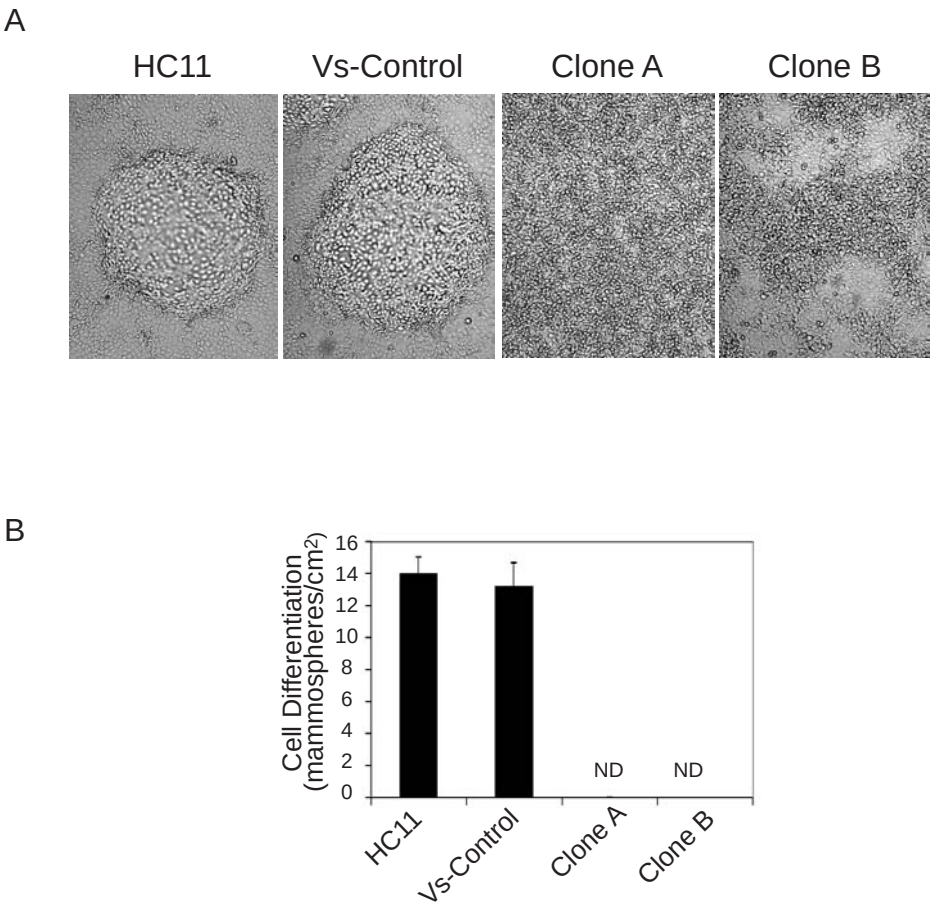


Figure 9. Antisense Jak2 blocked prolactin-induced differentiation of stably transfected HC11 clones. A, *Prolactin-induced HC11 cell differentiation is disrupted in Jak2 antisense expressing clones.* Parental HC11 cells, Vs-control clone, and *Jak2* antisense expressing clones A and B were incubated with prolactin for 4 days. Phase contrast images of representative fields show failure of mammosphere formation in *Jak2* antisense expressing clones. **B**, *Quantification of differentiation-suppressive effect of Jak2 antisense in stable HC11 clones.* The effect of stable expression of *Jak2* antisense on prolactin-induced HC11 cell differentiation was documented by counting of mammospheres after 4 days of treatment. Data are expressed as density of mammospheres in the cultures, and represent mean values (+SEM) of three independent experiments. ND indicates not detected. (Xie, LeBaron, Nevalainen, and Rui, 2002)

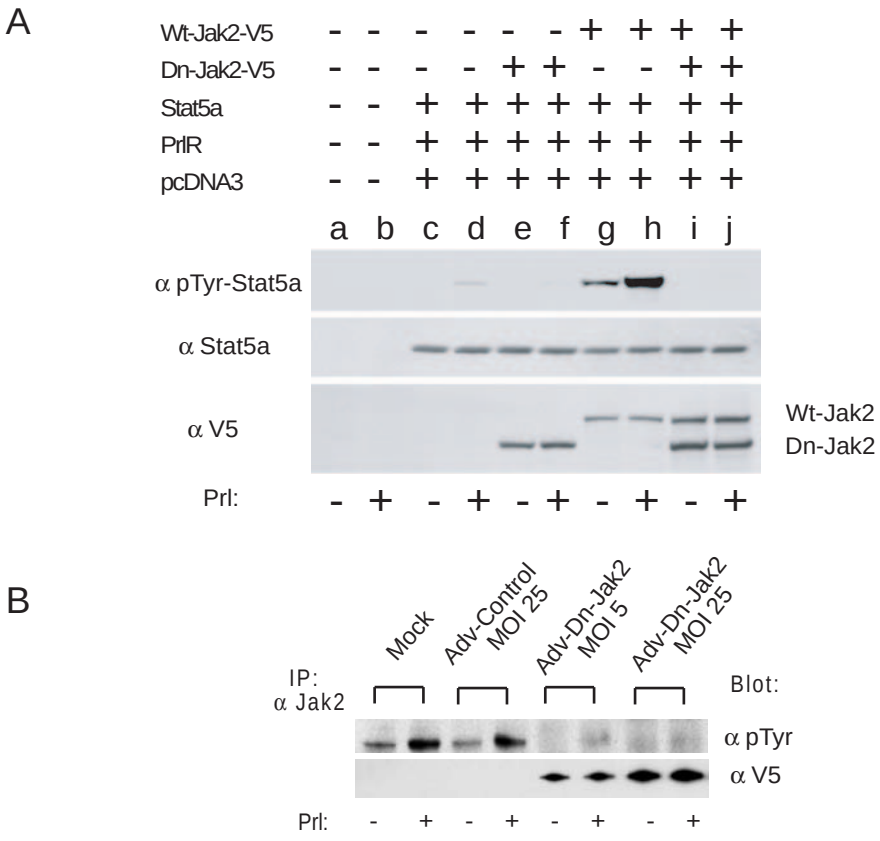


Figure. 10. Dominant-negative (Dn) Jak2 inhibited prolactin-induced HC11 cell differentiation. A, *Validation of efficiency of Dn-Jak2 construct by transient cotransfection in COS-7 cells.* COS-7 cells were cotransfected with plasmids encoding *prolactin receptor, Stat5a,* Wt-*Jak2*, and/or Dn-*Jak2* as indicated. Total amounts of DNA transfected were maintained constant by compensating with empty pcDNA3 vector. Parallel cultures were incubated in the presence (+) or absence (-) of prolactin (10 nM) for 30 min, and whole cell lysates were examined for tyrosine phosphorylated Stat5 (upper panel), Stat5a protein levels (middle panel), or V5-epitope tagged Wt-Jak2 or Dn-Jak2 (lower panel). **B**, *Validation of efficiency of adenoviral delivery of Dn-Jak2 to inhibit Jak2 activation in HC11 cells.* HC11 cells were either mock infected (no adenovirus), or infected with either Adv-Control (no insert; MOI 25), or with two increasing doses of Adv-Dn-*Jak2* (MOI 5 and 25). Following a 24-hour infection, cells were incubated with (+) or without (-) prolactin (10 nM) for 30 minutes. Jak2 was immunoprecipitated from whole cell lysates and basal and prolactin-induced Jak2 phosphotyrosine levels were determined by anti-pTyr immunoblotting (upper panel), and levels of V5 epitope-tagged Dn-Jak2 protein were determined in parallel samples by anti-V5 immunoblotting (lower panel). (Xie, LeBaron, Nevalainen, and Rui, 2002)

stimulation (Figure 10A, lanes a, b). When *Stat5a* and *PrlR* were co-transfected into COS-7 cells, modest but detectable prolactin-stimulated Stat5a phosphorylation was observed, presumably mediated by low levels of endogenous Jak2 (Figure 10A, lanes c, d).

This inducible Stat5a activation, or tyrosine phosphorylation, was inhibited by cotransfection with Dn-*Jak2*, which migrated with the expected size of 90 kDa in SDS-PAGE (Figure 10A, lanes e, f; top and bottom panels, respectively). Cotransfection of Wt-*Jak2* with *Stat5a* led to basal tyrosine phosphorylation of Stat5a that was markedly enhanced by prolactin treatment (lanes g, h). However, further cotransfection of Dn-*Jak2* with Wt-*Jak2* showed complete inhibition of both basal and prolactin-induced Stat5a activation (lanes i, j). This effect was not due to reduced levels of Stat5 or Wt-Jak2 as demonstrated by reprobing with anti-Stat5 or anti-V5 antibodies, respectively (Figure 10A, middle and lower panels). Furthermore, these and other immunoblotting experiments showed that Dn-Jak2 effectively inhibited Wt-Jak2 function, with respect to Stat5 activation, at equivalent protein levels, providing direct evidence for dominant rather than a simple competitive inhibitory effect of the kinase-deleted Jak2 mutant. Thus, the engineered Dn-Jak2 functioned as predicted.

Adenoviral Delivery of Dn-Jak2 into HC11 Cells Blocked Prolactin-Induced Differentiation

As the second approach to effectively inhibit the Jak2 tyrosine kinase in HC11 cells, we then generated a replication-defective adenovirus for high-efficiency gene delivery of Dn-*Jak2* into HC11 cells. Detailed description of this construct is presented in the *Materials and Methods* section. Functional testing of Adv-Dn-*Jak2* was first

carried out in HC11 cells using prolactin-stimulated Jak2 tyrosine phosphorylation as a readout. Cells were mock-infected, or infected with virus carrying no insert (Adv-control), or Adv-Dn-*Jak2* at increasing multiplicity of infection (MOI). Twenty-four hours later, cells were incubated with or without prolactin for 30 min, and Jak2 phosphotyrosine levels were examined. In HC11 cells, both basal and prolactin-activated Jak2 tyrosine phosphorylation were inhibited by Dn-Jak2 at MOI values of 5 and 25, whereas infection with control virus had no effect. Reblotting of samples for V5-tagged Dn-Jak2 protein verified specific and dose-dependent expression of Dn-Jak2 in HC11 cells (Figure 10B), and that Adv-Dn-*Jak2* was functional.

To determine whether Dn-Jak2 would block prolactin-induced differentiation of HC11 cells, cells were infected with or without Adv-Dn-*Jak2* as described and mammosphere formation in response to prolactin treatment was monitored. While prolactin-induced differentiation remained intact in mock and Adv-control infected cells, Dn-Jak2 effectively disrupted prolactin-induced mammosphere formation in HC11 mammary cells (Figure 11A). The effect of Dn-Jak2 was dose-dependent, and could not be attributed to general protein overexpression since infection with Adv-LacZ did not disrupt mammosphere formation (Figure 11B). Therefore, based on two independent approaches that involved either Dn-Jak2 or antisense to inactivate Jak2, we conclude that Jak2 tyrosine kinase activity, and presumably Stat5 activation, is required for terminal differentiation of mammary epithelial cells. To our knowledge, these data provide the first direct evidence that Jak2 tyrosine kinase activity is critical for prolactin-induced differentiation of mammary epithelial cells.

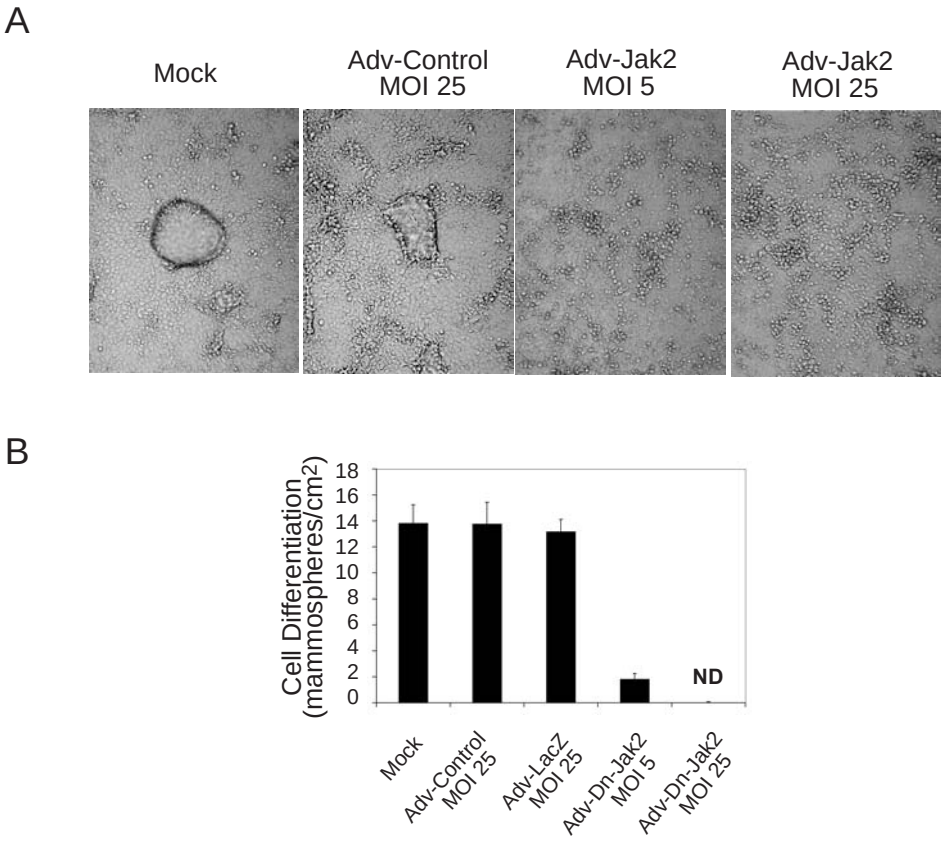


Figure 11. Dominant-negative (Dn) Jak2 inhibited prolactin-induced HC11 cell differentiation. A, *Adenoviral delivery of Dn-Jak2 blocks prolactin-induced HC11 cell differentiation.* HC11 cells were either mock infected, or infected with either Adv-Control (MOI 25), or with two increasing doses of Adv-Dn-*Jak2* (MOI 5 and 25) on Day 0 of a four-day prolactin-induced differentiation treatment. Phase contrast images of representative fields show failure of mammosphere formation in HC11 cells expressing Dn-Jak2. **B**, *Adenoviral delivery of Dn-Jak2 blocks prolactin-induced HC11 cell differentiation in a dose-dependent manner.* The effect of Dn-Jak2 on prolactin-induced HC11 cell differentiation was documented by counting of mammospheres after 4 days of adenoviral gene delivery. Data are expressed as density of mammospheres in the cultures, and represent mean values (+SEM) of three independent experiments. ND indicates not detected. (Xie, LeBaron, Nevalainen, and Rui, 2002)

Disruption of Jak2 Activity was Associated with Inhibition of Stat5a Tyrosine Phosphorylation in HC11 Cells

Transcription factor Stat5 is critical for terminal differentiation of mammary cells and for lactogenesis as determined from genetic studies in mice (Liu *et al.* 1997; Teglund *et al.* 1998). Furthermore, Stat5 is recognized to be a substrate of Jak2 in the context of the prolactin receptor complex in COS-7 cells (Gouilleux *et al.* 1994). To experimentally determine whether inhibition of Jak2 would block prolactin-induced Stat5 activation in HC11 cells, we tested the effect of Adv-Dn-*Jak2* on prolactin-induced Stat5 tyrosine phosphorylation. Stat5 activation was measured by protein immunoblotting of samples from whole cell lysates using a monoclonal anti-phosphotyrosine-Stat5 antibody (Figure 12A). Whereas prolactin-induced Stat5 activation was readily detectable in mock-infected cells or cells infected with Adv-control (lanes a-d), prolactin-induced Stat5 activation was inhibited in a dose-dependent manner in cells infected with Adv-Dn-*Jak2* (Figure 12A, lanes e-h, upper panel). Furthermore, inhibition of Stat5 activation correlated with Dn-Jak2 levels as detected by anti-V5 immunoblotting, and was not due to reduction in Stat5 protein levels (Figure 12A, middle and bottom panels, respectively).

Inhibition of prolactin-induced Stat5 tyrosine phosphorylation by Jak2 suppression was verified at the subcellular level by anti-phosphoTyr-Stat5 immunocytochemistry. HC11 cells infected with Adv-control and treated with prolactin showed marked nuclear tyrosine phosphorylation of Stat5, whereas infection of cells with Adv-Dn-*Jak2* markedly inhibited Stat5 phosphorylation (Figure 12B, panels 1 and 2).

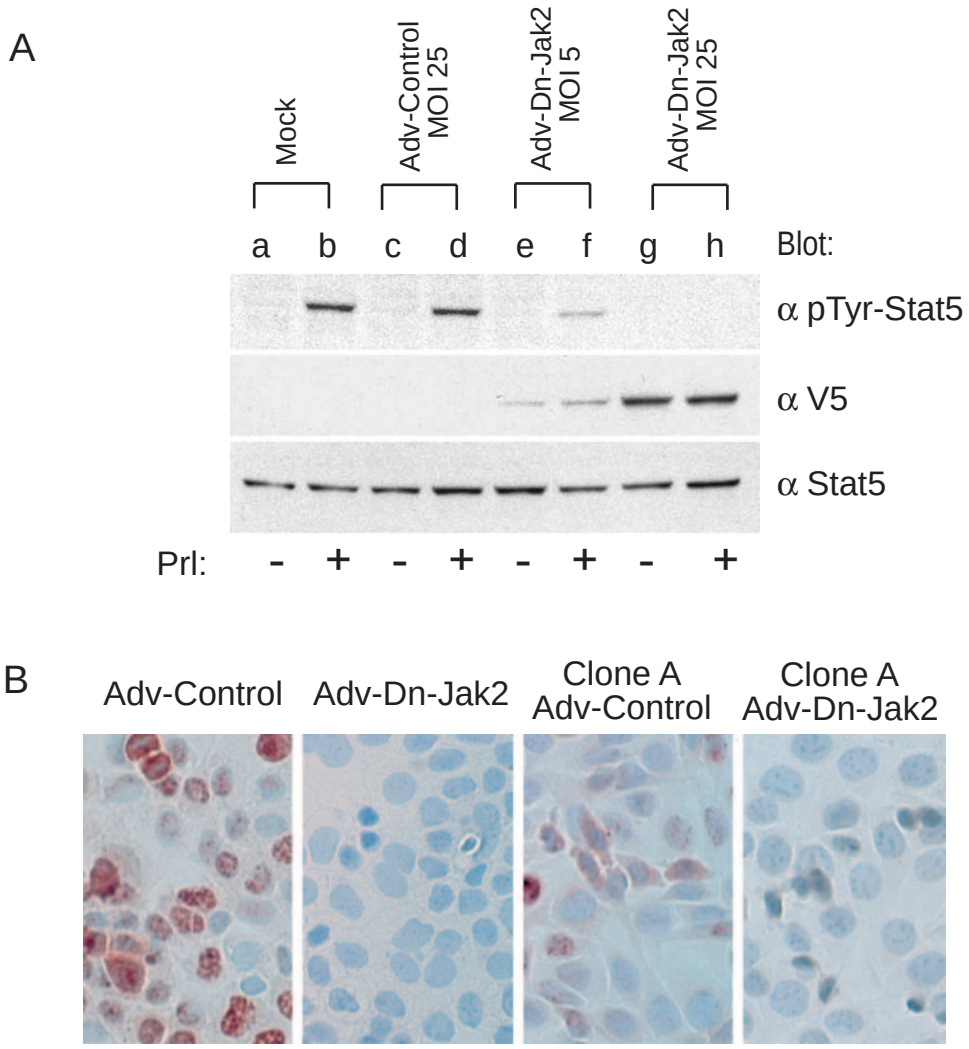


Figure 12. Dominant-negative Jak2 inhibited prolactin-induced Stat5 activation in HC11 cells. A, *Adenoviral delivery of Dn-Jak2 inhibits prolactin-induced Stat5 tyrosine phosphorylation in HC11 cells by anti-phosphotyrosine-Stat5 immunoblotting.* HC11 cells were either mock infected, or infected with either Adv-Control (no insert; MOI 25), or with two increasing doses of Adv-Dn-*Jak2* (MOI 5 and 25). Following a 24-hour infection, cells were incubated with (+) or without (-) prolactin (10 nM) for 30 min. Whole cell lysates were examined for tyrosine phosphorylated Stat5 (upper panel), V5-epitope tagged Dn-Jak2 (middle panel), or Stat5 protein levels (lower panel). **B**, *Adenoviral delivery of Dn-Jak2 inhibits prolactin-induced Stat5 tyrosine phosphorylation in HC11 cells by immunocytochemistry.* Parental HC11 cells or HC11 clone A, which stably expresses antisense *Jak2*, were infected with either Adv-Control (MOI 25; first and third panels, respectively) or Adv-Dn-*Jak2* (MOI 25; second and fourth panels, respectively). Following a 24-hour infection, all cells were exposed to prolactin for 30 min, followed by fixation and immunocytochemistry for activated Stat5 using anti-Stat5 pTyr antibodies. (Xie, LeBaron, Nevalainen, and Rui, 2002)

Likewise, HC11 cell clones stably expressing antisense-*Jak2* displayed only minor levels of prolactin-stimulated Stat5 phosphorylation as detected by anti-phosphoTyr-Stat5 immunocytochemistry (Figure 12B, panel 3; only clone A shown). Finally, infection of clone A with Adv-Dn-*Jak2* led to an even more pronounced inhibition of prolactin-induced Stat5 tyrosine phosphorylation (Figure 12B, panel 4). Therefore, both molecular approaches to inhibit Jak2 activity also inhibited prolactin-induced Stat5 activation. Collectively, these observations support the concept that the Jak2-Stat5 pathway is a differentiation-inducing axis in mammary epithelial cells.

Targeted Inactivation of Jak2 in HC11 Cells Resulted in a Hyperproliferative Phenotype

Terminal differentiation of cells is associated with exit from the cell cycle and inhibition of cell proliferation. To determine the effect of Jak2-dependent signaling on growth characteristics of HC11 cells, we compared the growth rates of HC11 clones A and B to those of parental HC11 cells and the sense-control clone. As shown in Figure 13A, HC11 clones A and B exhibited significantly higher growth rates than parental HC11 or the sense-control clone. In fact, the growth rates of clones A and B stably expressing *Jak2* antisense were approximately double that of parental or sense-control HC11 cells.

The increased growth rates of Jak2-suppressed clones A and B were also correlated with increased rates of mitosis during exponential growth, as visualized by propidium iodide staining of cells (Figure 13B). Flow cytometry of cells during exponential growth verified a markedly increased proportion of cycling cells with a corresponding reduction in cells in the G0/G1 phase in clones A and B when compared to

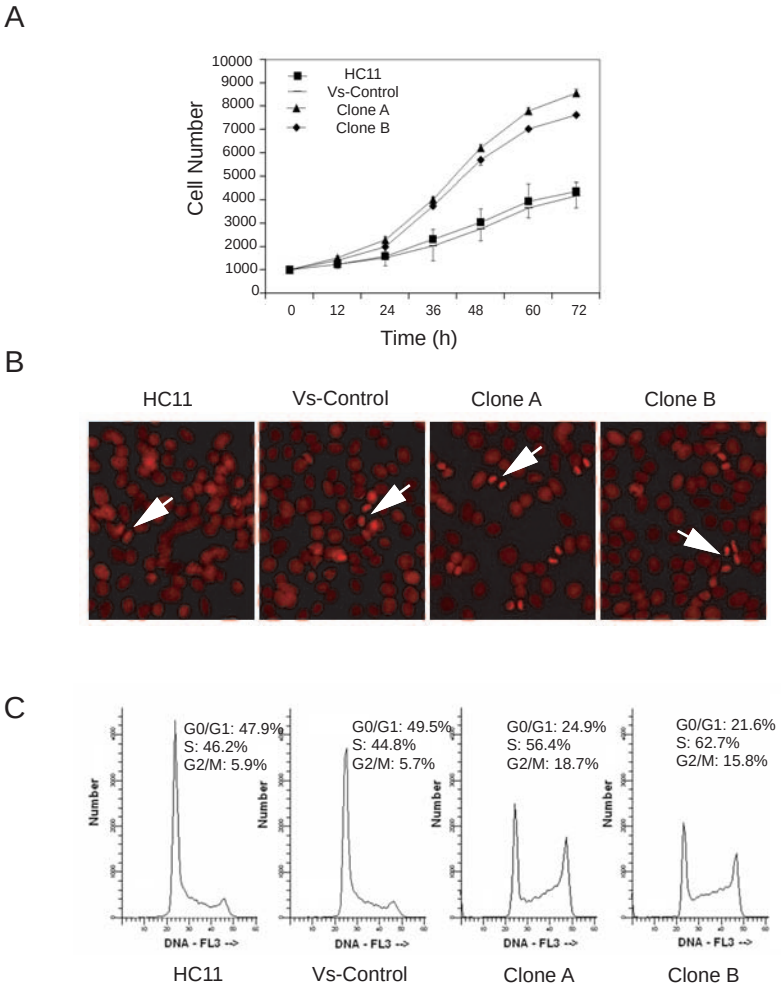


Figure 13. Suppression of Jak2 tyrosine kinase in Jak2 antisense expressing HC11 cell clones was associated with a hyperproliferative phenotype. **A**, *Increased growth rate in Jak2 antisense expressing HC11 clones A and B*. The growth rates of parental HC11 cells, vector-sense (Vs) control expressing control clone, and *Jak2* antisense expressing clones A and B were compared by plating cells at the same low density and following cell numbers over 72 h. Cell numbers were counted manually in a hemocytometer and the data represent means of three independent experiments (SEM indicated by bars). **B,** *Increased growth rate in Jak2-suppressed HC11 cells was associated with increased number of mitotic figures.* Exponentially growing cultures of parental HC11 cells, vector-sense (Vs) control expressing control clone, and *Jak2* antisense expressing clones A and B were stained with propidium iodide to better visualize dividing cells (indicated by arrows). **C**, *Increased growth rate in Jak2-suppressed HC11 cells was associated with increased number of cycling cells by flow cytometry.* Exponentially growing cultures of parental HC11 cells, vector-sense (Vs) control expressing control clone, and *Jak2* antisense expressing clones A and B were stained with propidium iodide and analyzed by flow cytometry to determine fraction of cells in the various stages of cell cycle. (Xie, LeBaron, Nevalainen, and Rui, 2002)

control cells (Figure 13C). Specifically, whereas almost 50% of parental or vector-sense control cells were in G0/G1, less than 25% of clones A and B were in G0/G1. For these cell cycle experiments, cells were harvested and measured at approximately 50% confluency. The data suggest that suppression of Jak2 tyrosine kinase activity is associated with increased cycling of HC11 cells and higher growth rate during exponential growth.

Targeted Inactivation of Jak2 in HC11 Cells Resulted in Decreased Contact Inhibition

We then examined the cell cycle characteristics of superconfluent cultures. Intriguingly, Jak2-deficient clones A and B consistently grew to a density nearly 3-fold greater than that of parental or vector sense-control HC11 cells (Figure 14A). Furthermore, during superconfluency, Jak2-deficient clones displayed reduced growth suppression and retained a markedly elevated S phase population compared to parental HC11 cells and vector sense-control cells (Figure 14B). Specifically, 15-21% of parental or vector sense-control cells were in S-phase during superconfluent conditions, whereas approximately 30% of antisense *Jak2* clones A and B remained in S-phase.

As an alternate method to inhibit Jak2 tyrosine kinase activity, we introduced Dn-*Jak2* into HC11 cells by adenoviral gene transfer and assessed its effect on cell cycling. Superconfluent cells that had been serum-deprived for 48 h were cycling only to a very low extent (< 4% in S or G2/M) in mock-infected or control virus-infected cells (Figure 15, left two panels). However, a dose-dependent increase in the fraction of cycling cells was observed in superconfluent, serum-deprived cells overexpressing Dn-Jak2 (Figure 15, right two panels). These observations were consistent with a general

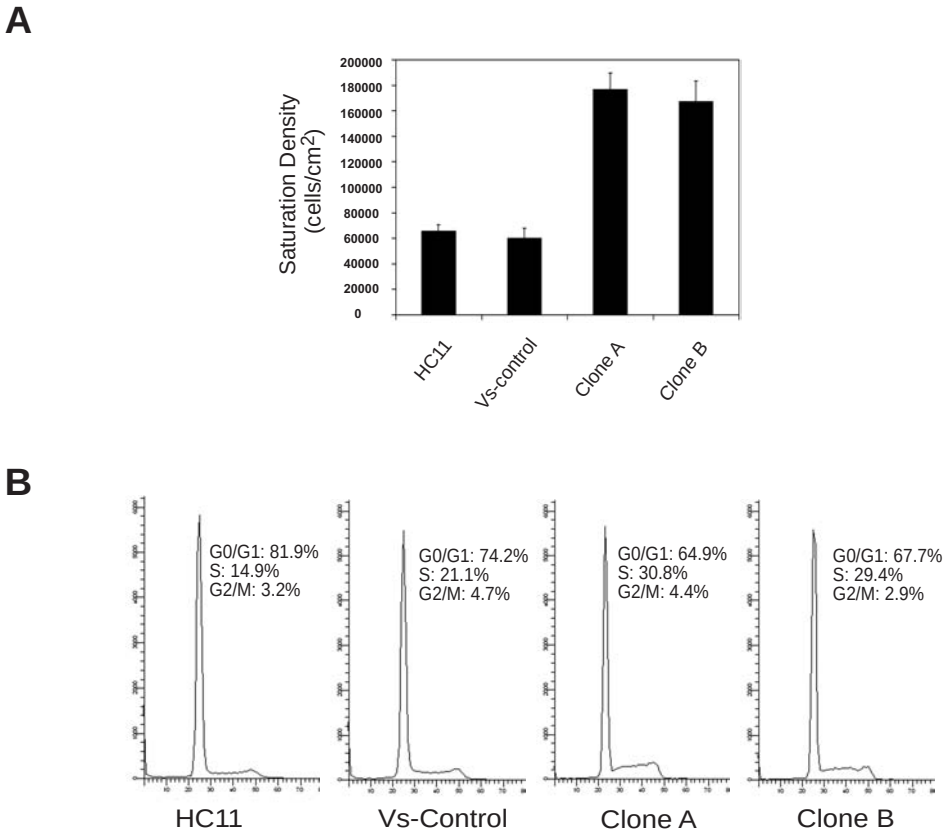


Figure 14. Suppression of Jak2 in stable Jak2 antisense expressing clones of HC11 cells was associated with reduced contact inhibition. A, *Increased saturation density of HC11 clones A and B.* Superconfluent cultures of parental HC11 cells, vector-sense (Vs) control expressing control clone, and *Jak2* antisense expressing clones A and B were counted to determine cell saturation density. Cell numbers were counted in duplicate in three independent experiments. Values represent mean cell number/cm^2 and bars represent SEM. **B**, *Increased fraction of cycling cells in superconfluent cultures of HC11 clones A and B.* Superconfluent cultures of parental HC11 cells, vector-sense (Vs) control expressing control clone, and *Jak2* antisense expressing clones A and B were stained with propidium iodide and analyzed by flow cytometry. (Xie, LeBaron, Nevalainen, and Rui, 2002)

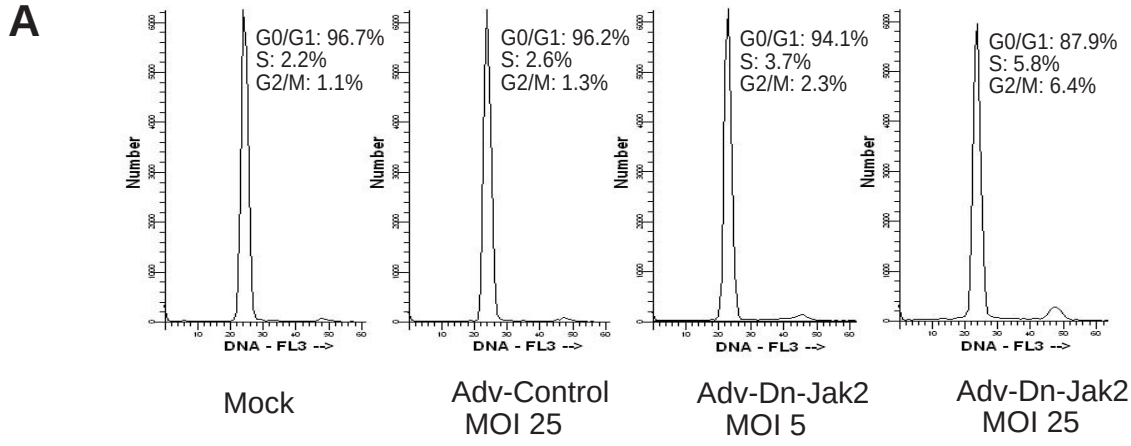


Figure 15. Suppression of Jak2 in stable Jak2 antisense expressing clones of HC11 cells was associated with reduced contact inhibition. A, *Dose-dependent increase in cycling cells in superconfluent cultures of HC11 cells exposed to Adv-Dn-Jak2*. Superconfluent cultures of parental HC11 cells were mock infected, or infected with either Adv-Control (no insert; MOI 25), or with two increasing doses of Adv-Dn-*Jak2* (MOI 5 and 25). Following 48 hours of infection and maintenance under serum-free conditions, cells were stained with propidium iodide and analyzed by flow cytometry. (Xie, LeBaron, Nevalainen, and Rui, 2002)

growth-suppressive effect of the Jak2-Stat5 pathway in HC11 cells. The data were also consistent with reduced contact inhibition following suppression of Jak2.

Inhibition of Jak2 Suppressed Apoptosis of HC11 Cells Induced by Anchorage-Independent Culture Conditions

The HC11 cell is a nontransformed mammary epithelial cell line that does not survive under anchorage-independent conditions. To investigate whether inhibition of Jak2 activity would affect the rate of apoptosis induced by culture under anchorage-independent culture conditions, we first examined apoptosis rates in HC11 cells stably expressing antisense *Jak2*. Parental HC11 cells, Vs-control cells, and clones A and B were cultured on 0.8% agar in normal growth medium, collected after 12 and 36 h, and assayed for apoptosis by flow cytometry.

The hypodiploid fraction of HC11 cells at 12 h was markedly lower in Jak2-suppressed clones A and B than in parental or vector control cells (Figure 16A), and although the number of apoptotic cells increased in both control cells and Jak2-suppressed cells over the next 24 h, Jak2-suppressed cells showed consistently reduced rates of apoptosis. Examination of apoptosis in parallel samples using TUNEL staining of fragmented DNA at 12 h verified reduced number of apoptotic cells in Jak2-suppressed clones, while phase-contrast and DAPI staining verified equal cell numbers in the selected fields (Figure 16B). However, extended cultures revealed that suppression of Jak2 levels in HC11 cells did not confer long-term survival under anchorage-independent culture conditions (data not shown).

Parallel studies of HC11 cells grown under anchorage-dependent conditions also provided evidence for reduced apoptosis following delivery of Dn-*Jak2* into confluent

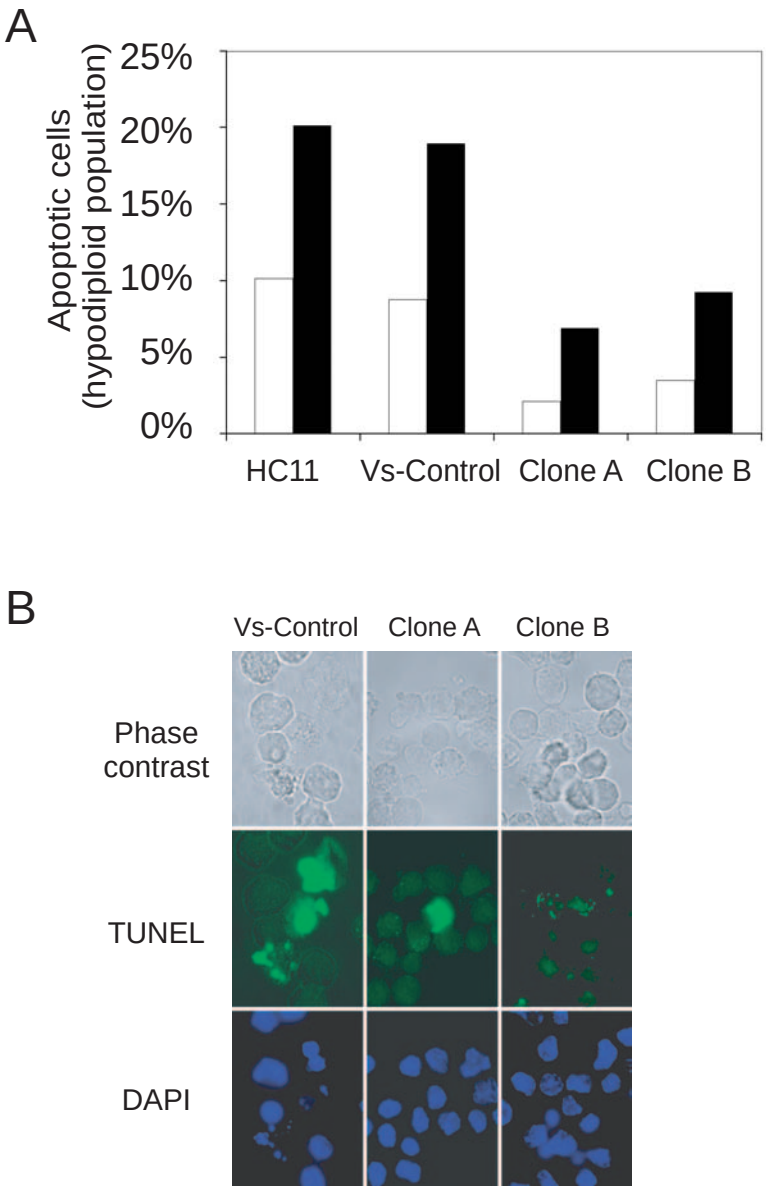


Figure 16. Suppression of Jak2 in HC11 cells was associated with reduced rate of apoptosis. A, *Reduced rate of apoptosis in HC11 clones A and B under anchorage-independent culture conditions.* Parental HC11 cells, vector-sense (Vs) control expressing control clone, and *Jak2* antisense expressing clones A and B were cultured on soft agar. After 12 h (open bars) or 36 h (filled bars), cells were harvested, stained with propidium iodide, and analyzed for hypodiploid, apoptotic cells by flow cytometry. A representative data set from two independent experiments is presented. **B**, *Reduced rate of apoptosis in HC11 clones A and B as visualized by TUNEL staining.* Reduced apoptosis rates in HC11 clones A and B under anchorage independent culture conditions for 36 h were verified by TUNEL staining of cells for DNA fragmentation (middle panel). Phase contrast (upper panel) and DAPI staining of DNA (lower panel) verified comparable number of cells in each representative field. (Xie, LeBaron, Nevalainen, and Rui, 2002)

cultures, as demonstrated by TUNEL staining of cells undergoing DNA fragmentation (Figure 17). Representative fields from cell cultures presented by phase-contrast, TUNEL, and DAPI staining, showed that a larger number of HC11 cells undergo apoptosis when infected with Adv-control compared to Adv-Dn-*Jak2* (Figure 17). We conclude from these experiments that suppression of Jak2-mediated signaling in HC11 cells inhibits cellular apoptosis under several culture conditions. Thus, the hyperproliferative phenotype of HC11 cells associated with suppression of Jak2 also involved anti-apoptotic elements.

The Hyperproliferative Phenotype Resulting from Jak2 Suppression in HC11 Cells was Associated with Constitutive Activation of Stat3

The observed hyperproliferative and undifferentiated phenotype of HC11 mammary epithelial cells with disrupted Jak2-Stat5 signaling was intriguing in light of the general loss of differentiation associated with progressing breast cancer cells. As mentioned in the *Introduction*, Stat3 has been shown to be an oncogene (Bromberg *et al.* 1999) and to be constitutively activated in human breast cancer (Garcia *et al.* 2001), therefore we examined the effect of Jak2 suppression on basal Stat3 activation in HC11 cells. Interestingly, western blot analysis of whole cell lysates from subconfluent HC11 cells indicated that Stat3 was constitutively active in hyperproliferative clones A and B, as determined by increased Stat3 phosphotyrosine levels in the absence of increased Stat3 levels (Figure 18A).

Similar results were obtained with introduction of Dn-*Jak2* into HC11 cells by adenoviral transfer. Dn-Jak2 also led to constitutive activation of Stat3 as measured by Stat3 phosphotyrosine and Stat3 protein immunoblotting (Figure 18B). The effect was

A

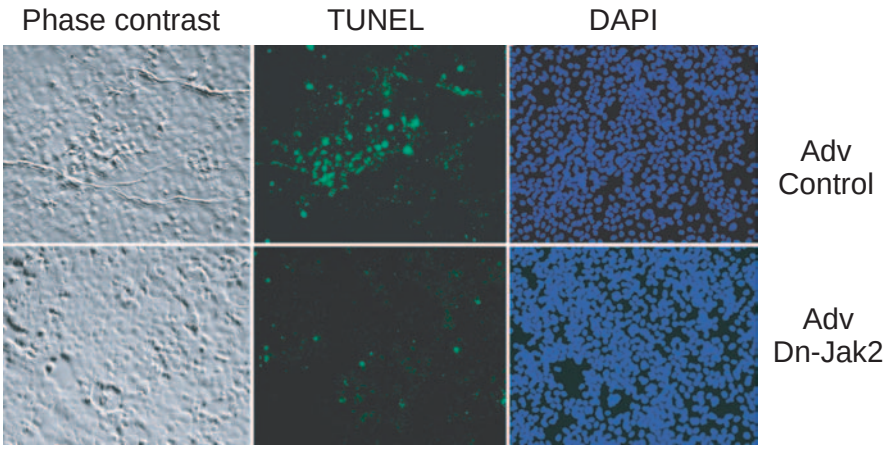


Figure 17. Suppression of Jak2 in HC11 cells was associated with reduced rate of apoptosis. A, *Reduced rates of apoptosis in superconfluent HC11 cells infected with Adv-Dn-Jak2.* Superconfluent cultures of parental HC11 cells cultured on plastic were infected with either Adv-Control (MOI 25), or with Adv-Dn-*Jak2* (MOI 25). Following 48 hours after infection, cells were TUNEL stained for DNA fragmentation (middle panels) and stained with DAPI (right panels). Left panels show phase contrast micrographies of the same fields. (Xie, LeBaron, Nevalainen, and Rui, 2002)

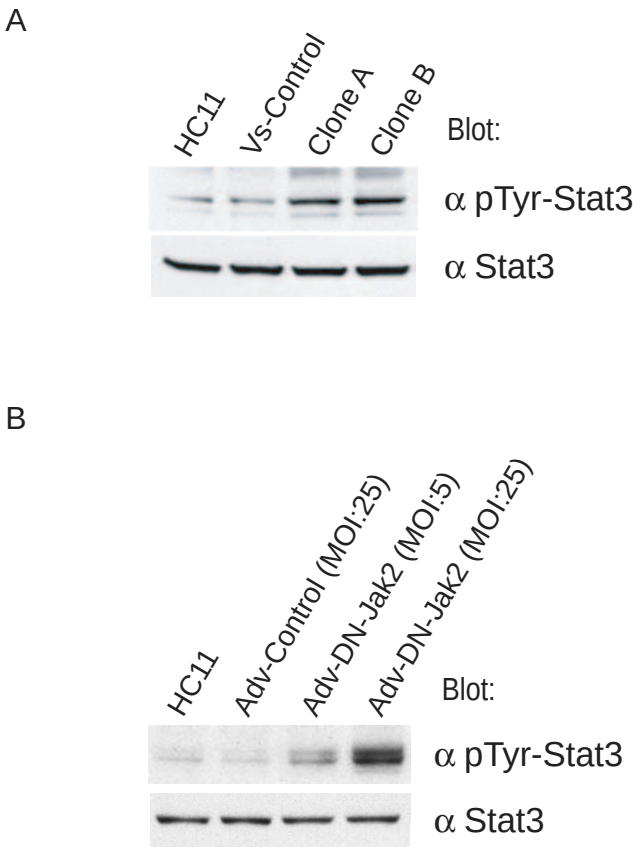


Figure 18. Suppression of Jak2 in HC11 cells was associated with constitutive activation of Stat3. A, *Constitutive activation of Stat3 in HC11 clones A and B.* Parental HC11 cells, Vs-control clone, and *Jak2* antisense expressing clones A and B were cultured under exponential growth conditions. Levels of tyrosine phosphorylated Stat3 were determined in whole cell lysates by immunoblotting with anti-pTyr-Stat3 antibodies (upper panel). Samples were reprobed for Stat3 protein levels to verify equal loading (lower panel). **B**, *Dose-dependent induction of constitutive Stat3 phosphotyrosine levels in HC11 cells infected with Adv-Dn-Jak2.* HC11 cells were either mock infected, or infected with either Adv-Control (MOI 25), or with two increasing doses of Adv-Dn-*Jak2* (MOI 5 and 25). Following a 24-hour infection, whole cell lysates were analyzed for Stat3 tyrosine phosphorylation (upper panel) and reprobed for Stat3 protein levels (lower panel). (Xie, LeBaron, Nevalainen, and Rui, 2002)

dose-dependent and was not induced by infection with control adenovirus. Based on two independent molecular approaches, we conclude that the hyperproliferative phenotype of Jak2-suppressed HC11 cells correlated with constitutive activation of Stat3. Thus, the possibility exists that constitutive activation of Stat3 was involved in the hyperproliferative phenotype of the cells associated with Jak2 suppression. Further investigation into the role of Stat3 in proliferation of HC11 cells, and the relationship between Stat3 activation and suppression of the Jak2-Stat5 pathway, are needed.

Discussion

The present study used adenoviral delivery of dominant-negative *Jak2* and stable expression of *Jak2* antisense mRNA to identify Jak2 kinase activity as a critical mediator of prolactin-induced differentiation of nontransformed HC11 mammary epithelial cells. The associated disruption of prolactin-induced Stat5 activation most likely represents the key molecular mechanism responsible for disrupting prolactin-induced differentiation. Furthermore, targeted suppression of Jak2 in HC11 cells led to a hyperproliferative phenotype, suggesting that Jak2 exerts a growth-inhibitory influence on normal mammary epithelial cells. Whereas suppression of Jak2 did not lead to transformation of HC11 cells, Jak2 suppression did, however, reduce contact inhibition and extend cell survival under anchorage-independent growth conditions. Stat3 was constitutively activated in hyperproliferative, Jak2-suppressed HC11 mammary epithelial cells, and this activation of Stat3, a known oncogene (Bromberg *et al.* 1999), may contribute to the hyperproliferative phenotype.

Jak2 as a Mediator of Prolactin-Induced Differentiation

Work performed by Dr. Hallgeir Rui originally identified Jak2 as the prolactin-associated tyrosine kinase in Nb2 lymphoma cells (Rui *et al.* 1992; Rui, Kirken, and Farrar 1994). While other Jak tyrosine kinases reportedly are not activated by prolactin receptors, numerous studies indicate that prolactin may activate other, non-Jak tyrosine kinases. These include tyrosine kinases Fyn (Clevenger and Medaglia 1994), Src (Berlanga *et al.* 1995), TEC (Kline, Moore, and Clevenger 2001), and focal adhesion kinase (Canbay *et al.* 1997). In most cases, temporal activation profiles suggest that prolactin activates Jak2 upstream of the non-Jak tyrosine kinases, but at least one report indicated that prolactin can activate Src independent of Jak2 (Fresno Vara *et al.* 2000). Consequently, the notion that all prolactin-induced effects are mediated by Jak2 tyrosine kinase activity may not be correct. Therefore, it was of particular importance to determine whether Jak2 mediates prolactin-induced differentiation of mammary epithelial cells.

The present study now provides direct evidence that Jak2, in fact, is critical for mammary epithelial cell differentiation. After establishing that prolactin activated Jak2, and not other Jaks, in HC11 cells, and that concentration-dependent induction of cell differentiation by prolactin correlated with Jak2 activation, two independent strategies were used *in vitro* for targeted suppression of Jak2. These included construction of two sets of novel reagents, a vector for stable or transient expression of a novel and specific *Jak2* antisense-mRNA, and a replication-defective adenovirus for efficient gene delivery of Dn-*Jak2*. Both sets of molecular tools were validated and determined to be effective in independent experiments, and both strategies independently demonstrated that Jak2

functional activity is critical for prolactin-induced differentiation by selectively disrupting mammosphere formation. Disruption of prolactin-induced differentiation was not associated with any general cytotoxic effects, since suppression of Jak2 by either strategy led to increased proliferation rates and reduced cellular apoptosis. Furthermore, the described molecular tools may be applied to determine whether Jak2 is critical for biological effects of other cytokine receptors in a variety of cell types.

Evidence That Jak2 Phosphorylates and Activates Stat5 in HC11 Cells

Transcription factor Stat5, and especially the Stat5a isoform, is critical for mammary gland differentiation (Liu *et al.* 1997). Stat5 is phosphorylated on a single tyrosine residue following prolactin receptor activation, and this modification causes Stat5 to dimerize, which in turn is needed for DNA binding and transcriptional regulation (Wakao, Gouilleux, and Groner 1994). Jak2 is presumed to mediate prolactin-induced tyrosine phosphorylation of Stat5 (Gouilleux *et al.* 1994), although Stat5 can also be phosphorylated by the Src tyrosine kinase (Kazansky *et al.* 1999). In the present study, we determined that suppression of Jak2 activity blocked prolactin-stimulated Stat5 tyrosine phosphorylation in HC11 cells, both by immunoblotting and by immunocytochemistry using an anti-phosphotyrosine-Stat5 antibody. Specifically, in Jak2-suppressed HC11 clones, Stat5 activation was significantly down-regulated. Furthermore, Stat5 activation was inhibited in a dose-dependent manner by Dn-*Jak2* delivered by adenoviral infection. These findings support a view of the Jak2-Stat5 pathway as a differentiation-inducing axis in mammary epithelial cells, presumably as a result of genes regulated by Stat5.

Targeted Inactivation of Jak2 Resulted in Hyperproliferative Phenotype of HC11 Cells

The Jak2-deficient HC11 clones showed significantly increased growth rates, loss of contact inhibition, and prolonged survival under anchorage-independent culture conditions. While suppression of Jak2 in HC11 cells was associated with loss of Stat5 activation, Stat3 became constitutively tyrosine phosphorylated. Constitutive activation of Stat3 was observed both in HC11 clones stably expressing the *Jak2* antisense construct and in cells overexpressing Dn-*Jak2* by adenoviral delivery, raising the possibility that the Jak2-Stat5 pathway normally inhibits Stat3 activation in HC11 cells. Interestingly, a similar mutual exclusion of Stat5 activation and Stat3 activation has been observed in mammary epithelial cells within the physiological setting of mammary gland involution (Groner and Hennighausen 2000). Weaning, or artificially induced milk stasis, rapidly shuts off Stat5 activation in lactating mammary epithelial cells, and Stat3 becomes activated instead (Li *et al.* 1997). While the importance of Stat3 activation in hyperproliferative HC11 cells remains to be determined, this observation is particularly intriguing in light of the established tumor-promoting role of Stat3 (Bromberg *et al.* 1999).

In summary, the work presented in this section demonstrated that Jak2 functional activity as a tyrosine kinase for Stat5 is critical for prolactin-induced differentiation of HC11 mouse mammary epithelial cells. Equally important, the data are also consistent with an overall growth-inhibitory role of the Jak2-Stat5 pathway in mammary epithelial cells. This notion is of direct relevance to mammary tumorigenesis, because the data suggest a tumor-suppressive role of the Jak2-Stat5 pathway in the mammary gland. In

general, cancer cells are characterized by enhanced growth and reduced levels of differentiation – traits exhibited in cells with defective Prl-Jak2-Stat5 signaling. This work established new insight into the function of Stat5 as a transcription factor in the mammary gland and indicated the need to determine the role of Stat5 regulated genes in growth and differentiation.

Materials and Methods

Hormones and Antibodies

Ovine prolactin (NIDDK-oPRL-19, AFP-9221A) and human prolactin (NIDDK-hPRL-SIAFP-B2, AFP-2969A) were kindly provided by Dr. A.F. Parlow under the sponsorship of the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, the National Institute of Child Health and Human Development, and the U.S. Department of Agriculture. Human epidermal growth factor (EGF) was purchased from Upstate Biotechnology (Lake Placid, NY). Dexamethasone and insulin were purchased from Sigma Chemical Company (St. Louis, MO). Monoclonal anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-phosphotyrosine-Stat5 antibody and polyclonal rabbit antisera to Jak1, Jak2, Jak3, Tyk2, Stat1, Stat3, and Stat5 were obtained from Advantex BioReagents (Conroe, Texas).

HC11 Cell ex vivo *Model of Mammary Epithelial Cell Differentiation*

The mouse mammary epithelial cell line HC11 (Ball *et al.* 1988) was grown to confluence in RPMI-1640 medium (Biofluids, Rockville, MD) supplemented with 10%

heat-inactivated fetal calf serum (Atlanta Biologicals, Norcross, GA), insulin (5 μg/ml), and EGF (10 ng/ml). Prior to hormone treatment, HC11 cells were starved for 48 h in medium lacking EGF and containing only 2% fetal calf serum. For studies of prolactin-induced differentiation, HC11 cells were then incubated with RPMI-1640 medium supplemented with 10% fetal calf serum, dexamethasone (0.1 μM) and insulin (5 μg/ml) in the presence or absence of ovine prolactin (10 nM) as indicated.

Expression vectors

Expression vector for murine Stat5a (pXM-Stat5a) was kindly provided by Xiuwen Liu and Lothar Hennighausen (National Institutes of Health, Bethesda, MD). Plasmid p3PRLR contains a 2.7-kb human *prolactin receptor* cDNA (kindly provided by Dr. Paul A. Kelly, Institut National de la Santé et de la Recherche Médicale, Paris, France) and subsequently cloned into the *EcoR*I site of pcDNA3 expression vector (Invitrogen, Carlsbad, CA) as described (Yamashita *et al.* 1998). Rat *Jak2* cDNA was originally cloned from an Nb2-SP cell cDNA library (Duhe *et al.* 1995), and a 3.7 kb ORF cDNA fragment was subcloned into the *Not* I and *Apa* I sites of pcDNA3 for expression studies.

Construction of V5/His Epitope-Tagged Wild-type (Wt) and Dominant-Negative (Dn) Jak2 Expression Vectors

In order to generate expression constructs encoding Wt-*Jak2* and a kinase-deleted, Dn-*Jak2* with C-terminal V5/His epitope tags that could be further subcloned into an adenoviral vector with limited selection of cloning sites, a two-step strategy used. First, a Wt-*Jak2* cDNA was cloned into the pcDNA3.1/V5-His vector (Invitrogen, Carlsbad, CA)

5' to the V5/His sequence. Second, irrelevant intervening sequence including *Jak2* UTR was deleted to generate Wt-*Jak2*-V5-His, and further deletion of the entire JH1 kinase domain was carried out to generate Dn-*Jak2*-V5-His. Specifically, rat *Jak2* cDNA containing the open reading frame was released from the pBK cloning vector (Duhe *et al.* 1995) by digestion with *Apa* I and *Not* I. This fragment was subcloned into the *Apa* I and *Not* I sites of the pcDNA3.1/V5-His vector. In order to remove intervening sequence to generate V5-His tagged Wt-*Jak2*, and in the case of Dn-*Jak2* also to remove the JH1 domain, a modified pCR2.1 vector (Invitrogen) was used from which we had removed the *Kpn* I site. The *Kpn* I site in pCR2.1 vector was deleted by *Kpn* I digestion, blunt-ending by T4 DNA polymerase, relegation with T4 DNA Ligase, and confirmation by *Kpn* I re-digestion. The *Jak2*-V5-His pcDNA3.1 construct was digested with *Not* I and *Pme* I and the resulting fragment was subcloned into the *Not* I-*Pme* I site of the modified pCR2.1 vector.

High-fidelity PCR was used to 1) generate a short fragment A spanning from upstream of the *Avr* II site within the JH1 domain and containing a 3' *Sac* II restriction site after the last *Jak2* codon, and, 2) generate a second short fragment B spanning from upstream of the *Kpn* I site within the JH2 domain to introduce a 3' *Sac* II restriction site within the hinge region located between the JH1 and the JH2 domains. To generate a contiguous Wt-*Jak2*-V5-His construct, irrelevant sequence was released from the original *Jak2*-pcDNA 3.1/V5-His vector by *Avr* II and *Sac* II digestion, and replaced by correspondingly digested high-fidelity PCR fragment A. For the Dn-*Jak2* construct, the original *Jak2*-pcDNA 3.1/V5-His vector was digested with *Kpn* I and *Sac* II and the released fragment was replaced by the high-fidelity PCR fragment B. Finally, the Wt-

Jak2-V5-His and the Dn-*Jak2*-V5-His genes were released by *Not* I and *Spe* I digestion, blunted by T4 DNA polymerase and cloned into blunt-ended *Not* I and *Xba* I restriction sites of the pcDNA3 vector. All cloning was verified by DNA sequencing.

Antisense Jak2 Construct and Generation of Stably Expressing HC11 Clones

After testing several alternatives, an effective and specific *Jak2* antisense oligonucleotide was determined as 5'-TGT CTT CAA AAG CAC CAG AAA ATC CTA GGG CAC CTA TTC TCA TGT TGG GTA-3'. This targeting sequence was verified to be unique to *Jak2* by searching the NCBI GenBank database. The sequence targets a region of *Jak2* mRNA encoding the amino acid sequence PNMRIGALGFSGAFEDR within the hinge region between the JH1 and JH2 domains (Duhe *et al.* 1995). The sense control nucleotide sequence is 5'-TAC CCA ACA TGA GAA TAG GTG CCC TAG GAT TTT CTG GTG CTT TTG AAG ACA-3'. Both the antisense and sense *Jak2* DNA were generated by PCR, and a 5' *Eco*R V flanking site was introduced for orientation identification and subsequent cloning into pCR2.1. The sense and antisense *Jak2* DNA were released by *Hin*D III and *Not* I digestion, and were subcloned into the pcDNA3 vector at the multiple cloning site. The purified antisense and sense *Jak2* cDNA-pcDNA3 constructs were transfected into HC11 cells using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA), and stable clones were isolated after 10 days of treatment with 300 μg/ml G418 (Invitrogen, Carlsbad, CA). The clones were validated with Neomycin resistance gene PCR product analysis.

Cell Culture and Transient Transfections

Construct expression and functional tests were performed by transient transfection of COS-7 cells (ATCC, Manassas, VA). COS-7 cells were grown in RPMI-1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively) at 37 °C with 5% CO_2. Sub-confluent COS-7 cells in 6-well plates were transfected using LipofectAMINE 2000 according the manufacturer's protocol and were kept without fetal calf serum for 24-48 h followed by stimulation of 10 nM prolactin for 30 min. The harvested cell pellets were frozen on dry ice and stored at –80 °C.

Growth medium for T-47D human breast cancer cell line (American Type Culture Collection [ATCC]; Rockville, MD) was DMEM (Biofluids division of Biosource International, Camarillo, CA) containing 10% fetal calf serum (Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine (Biofluids division of Biosource International, Camarillo, CA; 50 IU/ml), and penicillin-streptomycin (Biofluids division of Biosource International, Camarillo, CA; 50 g/ml, at 37°C with 5% CO_2.

Adherent mammary epithelial cells were propagated by enzymatically dissociating them from the plastic substrate with trypsin-tetrasodium ethylenediaminetetraacetate (EDTA) buffer (0.5 mg/ml porcine trypsin, 0.2 mg/ml EDTA in Hanks' Balanced Salts with phenol red; Biofluids division of Biosource International, Camarillo, CA). The trypsinate was inactivated with fresh growth medium and the cells replated at the appropriate transfer ratio, primarily 1:3.

When necessary, cells were frozen as follows: after trypsinization, cells were pelleted by centrifugation for 1 min at 1,500 x *g*. The supernatant was removed and the cells resuspended in freezing medium containing DMEM, 10% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO), 10% fetal calf serum, 2 mM L-glutamine (50 IU/ml), penicillin-streptomycin (50 g/ml). The cell suspension was aliquoted and brought slowly to -80°C, then transferred to liquid nitrogen storage containers.

Protein Solubilization, Immunoblotting, and Immunoprecipitation

For protein solubilization, the cell pellets were solubilized in lysis buffer (10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30mM sodium pyrophosphate, 50mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 1 µg/ml pepstatin A, and 2 µg/ml leupeptin). Cell lysates were rotated end-over-end at 4 °C for 60 min, and insoluble material was pelleted at 12,000 × *g* for 30 min at 4 °C. For immunoprecipitations, the protein concentrations of clarified tissue lysates were determined by simplified Bradford method (BioRad Laboratories, Hercules, CA). Clarified lysates corresponding to 3.5 mg of total protein were immunoprecipitated by rotation for 2 h at 4 °C with the appropriate antibodies. Antibodies were captured by incubation for 60 min with protein A-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ), and washed 3 times in 1 ml of lysis buffer. Immunoprecipitated proteins were dissolved in 1.1 × loading buffer containing reducing agent. The proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting as described previously using polyvinylidene difluoride membranes (Millipore, Bedford, MA) and horseradish-peroxidase conjugated

secondary antibodies in conjunction with enhanced chemiluminescence substrate mixture (Amersham Pharmacia Biotech) and exposed to x-ray film.

Dominant-Negative Jak2 *Recombinant Adenovirus*

A replication-defective human adenovirus (Ad5) carrying Dn-*Jak2* was generated using the AdEasy Vector system (Quantum Biotechnologies, Carlsbad, CA). Briefly, the sequence-verified and expression-confirmed Dn-*Jak2*-V5/His expression cassette was released by *Apa* I and *Not* I digestion. Before *Not* I digestion, the *Apa* I digested ends were blunt-ended by *T4* DNA polymerase. The fragment was then subcloned into the *Not* I and *EcoR* V sites of Adv-shuttle vector. After creating a recombinant Dn-*Jak2*-V5/His Adv gene using pAdEasy, the virus was packaged in QBI-293A cells and subsequent plaques were isolated. Expression of Dn-Jak2 was verified by western blotting using anti-V5 and anti-Jak2 antibodies. The selected recombinant virus was expanded, purified, and titered in QBI-293A cells as per the manufacturer's recommendation. Two additional recombinant adenoviruses were used as controls: Adv-*LacZ*, which specifies a nuclear-localized form of β-galactosidase and Adv-Control that does not express a protein.

Flow Cytometry

HC11 cells were washed once in PBS, trypsinized, pelleted at 1,000 x g, and washed once in 5 ml cold PBS. After a second centrifugation, cells were resuspended in 0.5 ml cold PBS and fixed by dropwise addition of 1.5 ml cold 100% ethanol, while slowly vortexing the cell suspension. After having been fixed for 1 h at 4°C, cells were

stained with 100 μg/ml propidium iodide (Boerhinger-Mannheim, Indianapolis, IN) and treated with 100 ng/ml RNase A (Invitrogen, Carlsbad, CA) for 30 min at 37 °C. The cells were measured for DNA content by flow cytometry using a Coulter EPICS XL Cell Analyzer (Beckman-Coulter, Brea, CA).

TUNEL Assay

In situ detection of apoptotic cells was performed using Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL). Cells were air-dried on glass slides and fixed in 4% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 1 min on ice. The slides were rinsed with PBS several times and the samples were then processed for TUNEL labeling using the fluorescein-based *In Situ* Cell Death Detection Kit (Boerhinger-Mannheim, Indianapolis, IN) according to the manufacturer's instructions. Samples were rinsed 3 times with PBS, mounted, and analyzed under a fluorescent microscope.

Anchorage Independent Survival Analysis

Confluent HC11 cells were trypsinized into a single cell suspension. A total of 700,000 cells per group were plated in 150-mm culture dishes that had previously been coated with 0.8% agarose. Cells were collected at various time points, washed in PBS, and cell aggregates were dispersed by trypsinization. Parallel samples were analyzed for apoptosis by TUNEL staining and flow cytometry for hypodiploid cells.

Anti-phosphotyrosine-Stat5 Immunocytochemistry

HC11 cells were fixed in 4% paraformaldehyde-PBS at room temperature for 20 min. A cell scraper was used to gently detach the cells in PBS. The detached cells were stretched into monolayer sheets in warm PBS, and adhered to poly-lysine pretreated glass slides. Before immunocytochemical staining, sample slides were pretreated with an antigen unmasking procedure by boiling in an antigen-retrieval solution for 10 min. The slides were incubated at 4°C overnight by using a 1:2,000 dilution of the primary anti-phosphoTyr-Stat5 monoclonal antibody AX1 (Advantex Bioreagents, Conroe, TX). For secondary detection the Histomouse Kit (Zymed, South San Francisco, CA) was used, and active Stat5 was visualized with aminoethyl carbazole and counterstaining with hematoxylin. The prolactin treated COS-7 cells cotransfected with Stat5a and prolactin receptor expression construct were used as positive control, and subtype-specific mouse IgG and PBS were used for negative controls (not shown).

CHAPTER II:

DEVELOPMENT OF A METHOD FOR GENOME-WIDE IDENTIFICATION OF STAT5-CHROMATIN INTERACTION SITES

Introduction

Broad evidence presented in the *Background* section, and extended in the previous *Results* chapter, suggest an important role of the Jak2-Stat5 signaling pathway in maintaining breast epithelial cell differentiation and inhibiting cell proliferation. Identifying Stat5 target genes is needed to better understand the postulated role of Stat5 as a suppressor of breast cancer progression. The following section outlines new methodology that the author has developed and validated for genome-wide identification of Stat5 interaction sites. In this particular section of the *Results* the materials and methods have been integrated, because the overall intent was to develop and optimize novel methodology. Briefly, the new technology incorporates elements of chromatin immunoprecipitation protocols (Agarwal, Avni, and Rao 2000; Ren *et al.* 2000) with efficient cloning, sequencing, and genome-localization of captured DNA as well as experimental validation of the identified sequences.

While the specific protocol is detailed in the *Materials and Methods* section, a general experimental methodology for the cloning of Stat5-chromatin interaction sites is described below: (and graphically illustrated in Figure 19) First, activation of Stat5 allows binding to its respective *in situ* genomic response elements located along the DNA helix. Next, Stat5 is then covalently cross-linked to the DNA by the addition of formaldehyde directly to the medium of the cell culture. Third, after rinsing and

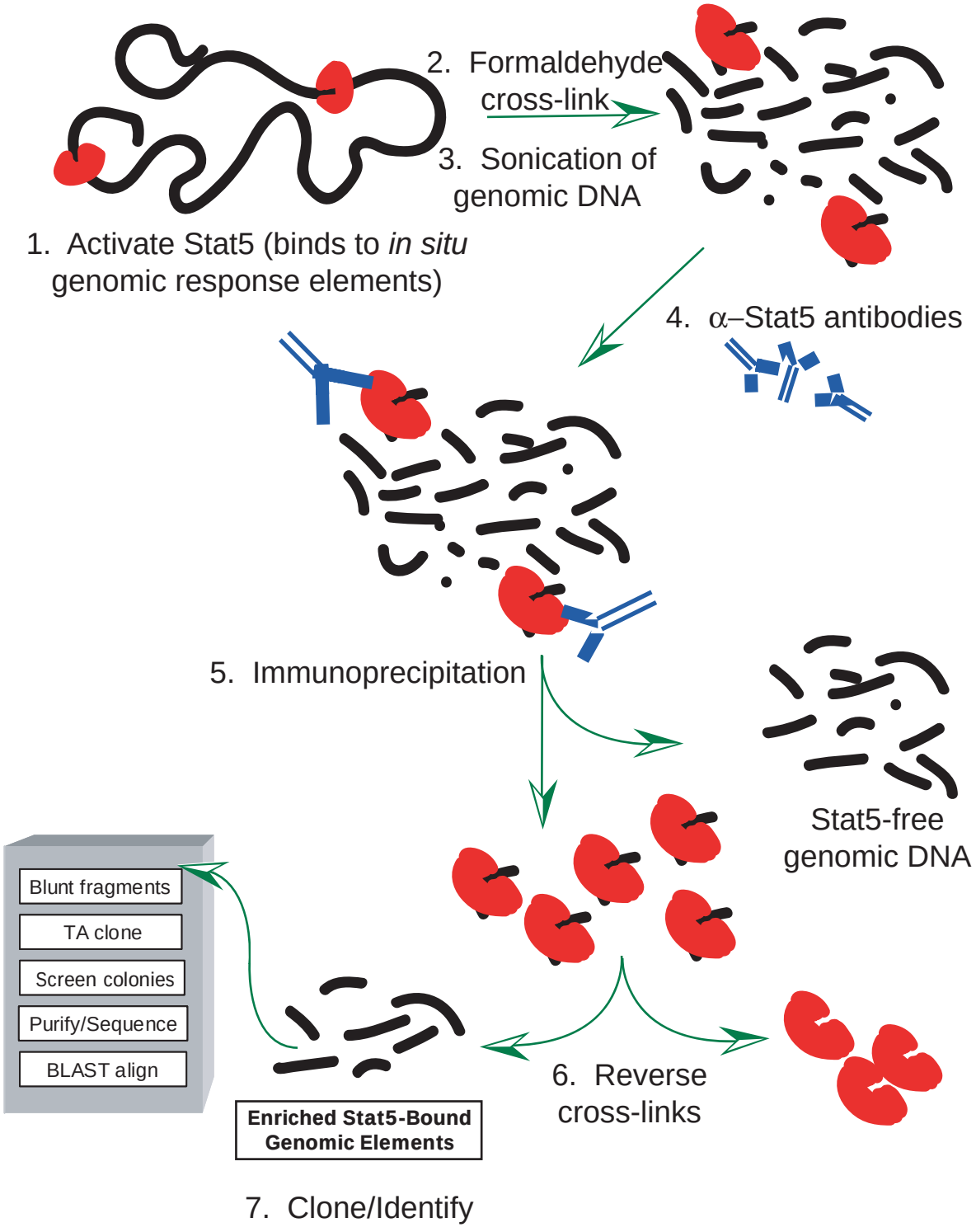


Figure 19. Method for capture of Stat5-bound genomic elements. The enriched pool can be used for cloning or other analysis studies.

collecting the cells, the cell pellets are resuspended in a cell-lysis buffer and sonicated under optimized parameters to yield approximately 400 base pair fragments, which are easily manipulated in subsequent steps. Fourth, specific Stat5 antibodies, which associate with the activated Stat5 dimers (which still have their respective genomic response element still attached), are added and then immunoprecipitated with protein A Sepharose beads. The sixth step is to reverse the cross-links and recover the DNA, yielding a final enriched pool of Stat5 binding elements, which can be further characterized in a number of ways. For example, the author has developed a method that allows the cloning into a standard bacterial expression vector after manipulation of the DNA fragment. The cloning process has been further streamlined by quantifying the inserts present in each positive clone by performing direct PCR on the bacterial colonies, thereby eliminating expensive and time-consuming plasmid mini-preps. Following purification and sequencing, the fragments are localized within the human genome via BLAST analysis to determine the site of Stat5-chromatin interaction in a genome-wide manner.

Additionally, the author has also established independent, but parallel, methodologies to validate the cloned Stat5-chromatin interaction sites that are detailed in the validation subsection of this results section.

Results/Discussion

Initial Cell-free Developmental Experiments

Conceptual Development

One of the first steps undertaken in the development of this method was to determine the feasibility of immunoprecipitating specific DNA elements bound by Stat5. In order to initially assess whether a method could be devised for immunoprecipitation, experiments were first performed in an *in vitro* and cell-free setting. This allowed a highly controlled environment so that a known DNA target for Stat5 could be investigated.

Specifically, COS-7 cells were transiently transfected with expression plasmids for *PrlR*, *Jak2*, and *Stat5a* using FuGENE 6 (Roche Diagnostics Corporation, Indianapolis, IN - see experimental details in the *Materials and Methods* section). After stimulation of the cells with prolactin, activated Stat5 was harvested by using an Electrophoretic Mobility Shift Assay (EMSA) lysis protocol compatible with DNA binding studies.

A specific Stat5-responsive reporter plasmid was used to provide DNA known to bind Stat5. The *β-Casein* promoter within this construct will specifically bind Stat5 as shown in previous reporter gene assays (Stocklin *et al.* 1996; Yamashita *et al.* 2001; Yamashita *et al.* 1998). In this simplified experiment, active Stat5 was mixed with the plasmid and allowed to bind. The plasmid was then digested to produce Stat5-binding fragments and non-binding fragments and run on an agarose gel to determine if there was

a change in migration of particular bands as a result of the presence of Stat5 still associated with the binding site.

Specifically, 22µl of COS-7 cytoplasmic extracts containing activated Stat5 were added to 10 µg of β-casein/luciferase plasmid (1 µg/µl) and 1.0 ml of EMSA binding buffer (50 mM Tris-HCl, pH 7.4, 25mM $MgCl_2$, 5 mM DTT, and 50% Glycerol). Lysates were determined to have a total protein concentration of 20 µg/µl, and an optimal ratio of 10 µg of total protein per 0.001 µg of probe was used to determine the amount of protein to be added. Furthermore, a ratio of 10 µg of total protein to 30µl of binding buffer was used to calculate the amount of binding buffer to be added to the reaction mixture. The binding reaction was then allowed to proceed for 30 minutes at room temperature with rotation.

After Stat5 was allowed to bind to its specific promoter, the plasmid was then digested to look for changes in band migration in samples where Stat5 was added. Parallel samples of the plasmid were digested with several restriction endonucleases to give different size fragments. In an *Ssp*I digested plasmid the band that contained the Stat5 binding site migrated slower than the control digested plasmid without activated Stat5 added (data not shown). This initial experiment provided significant insight to the feasibility of the proposed protocol and, therefore, required further, stringent testing.

In order to increase the sensitivity and specificity of the assay, PCR was used to identify the binding site for Stat5 within the plasmid. PCR primers were designed to the promoter flanking the Stat5 binding sites within the plasmid and were synthesized as follows: 5'- TCA TTG CCA CAT AGG TGA GG - 3' and 5'- GAC ATG CTA ATT TGT GGT TCG - 3' to give a 263 base pair (bp) product. Briefly, parallel samples of the

plasmid were digested with the endonucleases *Ssp*I or *Nde*I and *Xba*I in 40 µl reactions. The products of the digestions were then run on a 0.8% agarose gel (Figure 20). The bands were then independently excised and recovered using the QIAEX II ™ agarose gel extraction kit (Qiagen, Valencia, CA) per the manufacturer's protocol.

Independent PCRs were performed on each band recovered from the digestion to determine the site of Stat5 binding within the plasmid. As can be seen in Figure 21, Stat5 associates with the region of the plasmid that correlates with band #1 of the *Ssp*I digestion and not with the other regions of the plasmid. In the *Nde*I/*Xba*I digestion Stat5 associates with band #2. Note the positive control in lane #1, which was performed on undigested plasmid and the no template negative control in lane #2. This verified themotif of the plasmid construct and also provided a tool for future use in semi-quantitative assays as determined by PCR amplification.

Antibody Selection/Immunoprecipitation Optimization

As a next step in the development of the method, the conditions for immunoprecipitation were optimized, including experimentally testing the efficiency of several specific anti-Stat5 antibodies. As before, EMSA lysate containing activated Stat5 was mixed with *β-casein* promoter-*luciferase* plasmid and allowed to bind. The plasmid was then digested and anti-Stat5 or negative control antibodies were added to parallel samples. In each sample a total of 1 µg of antibody was added and normal rabbit serum (NRS) was used as a non-specific negative IP control. The samples were incubated for 2 hours on a rocking platform at 4°C to allow the antibody to bind to its specific epitope. Antibody/Stat5 immune complexes were then pulled down by immunoprecipitation after

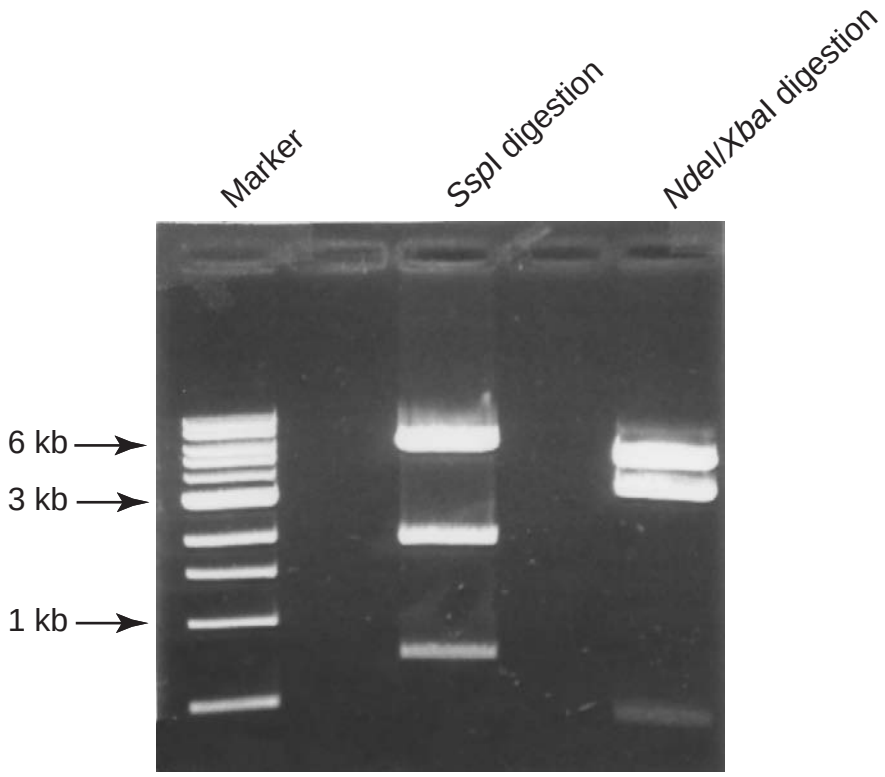


Figure 20. Endonuclease digestion of β-casein/luciferase plasmid. After digestion, the bands were excised and recovered individually. The products were used in future experiments to specifically determine the site of Stat5 association with the plasmid.

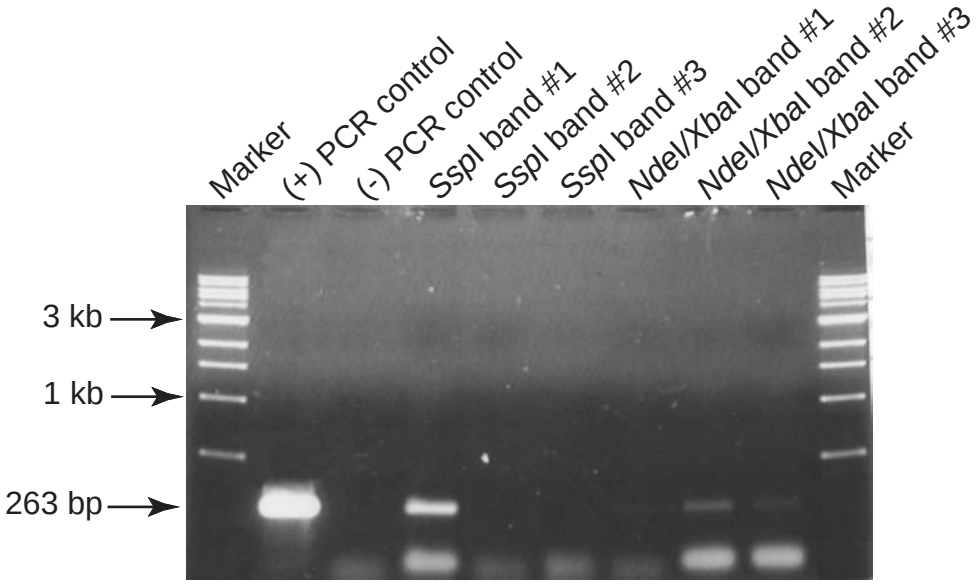


Figure 21. Construction of the β-casein/luciferase plasmid and identification of the Stat5 response element. PCR amplification with primers that flank the Stat5 binding site establish the location of the Stat5 response element. Note the PCR positive control performed on undigested plasmid and the 263 bp product. Negative PCR control was performed with no template added. The semi-quantitive assay also provides a tool for future optimization and analysis with respect to Stat5 association with DNA.

1 h of incubation with 40 µl Protein A Sepharose beads (Amersham Pharmacia, Piscataway, NJ). Before addition, protein A Sepharose beads were prepared by adding 240 µl of Protein A Sepharose beads to a 15 ml conical tube and were spun at 2000 RPM for 5 minutes and supernatant was removed. The beads were resuspended and washed twice in 5 ml of TE (pH 8.0), 0.1% BSA, and 0.1% NaN_3, spun, and supernatant removed. The beads were reconstituted in a final volume of 250 µl of the same buffer. The immune complexes and beads were collected by centrifugation at 14,000 RPM for 2 minutes and supernatant was removed. To elute the immune complexes from the Protein A Sepharose beads, the pellets were resuspended in 50 µl of TE and boiled for 10 minutes. Next, the samples were spun at 14,000 RPM for 2 minutes to pellet the beads and the supernatant was transferred to a separate tube.

A PCR was set up to semi-quantitatively determine the effectiveness of different Stat5 antibodies to immunoprecipitate the Stat5/*β-casein* promoter complexes. As can be seen in Figure 22 there was a noticeable difference in the ability of the various anti-Stat5 antibodies to immunoprecipitate the immune complexes. In lane #1 the plasmid with active Stat5 bound to the response element was digested by *Ssp*I restriction endonuclease and was used as a template for the positive control PCR reaction (no immunoprecipitation). Lane #2 was a no-template, negative PCR control. Lane #3 was immunoprecipitated with a non-specific normal rabbit serum (NRS) as a negative IP control. Lane #4 was a specific Stat5 antibody directed to the N-terminal portion of the molecule. Lane #5 was a specific Stat5 antibody synthesized to the C-terminal portion or transactivation domain of the Stat5 molecule. As is evident from Figure 22, it is important to note that the N-terminal Stat5 antibody is not only the strongest antibody

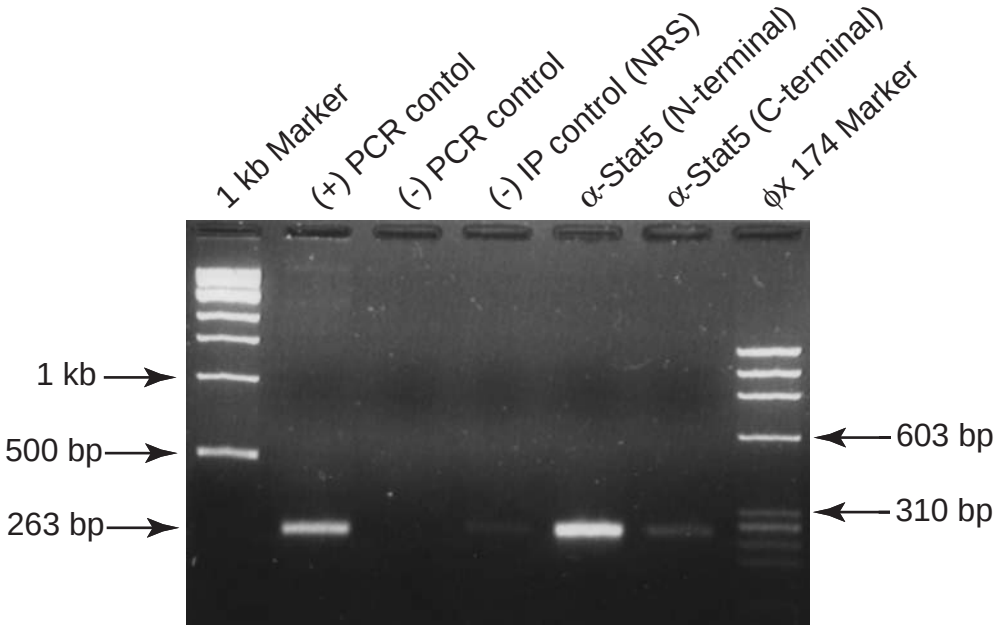


Figure 22. Optimization of immunoprecipitation of Stat5 bound to a DNA response element. Active Stat5 was added to β-casein/luciferase plasmid and was allowed to bind. The plasmid was then digested with *Ssp*I endonuclease. The products were then immunoprecipitated with the antibody as indicated. Primers designed to the Stat5 binding site were then used to amplify products for a semi-quantitative analysis of immunoprecipitation efficiency. N-terminal Stat5 antibodies were most effective in immunoprecipitating Stat5-bound DNA complexes. Postive PCR control was non-immunoprecipitated plasmid that had been digested as other samples. Negative PCR control was a no template reaction.

used for immunoprecipitation, but is also the best choice biologically. Post-translational modifications, as well as alternate gene splicing, of Stat5 molecules *in vivo* has been documented to generate species of Stat5 molecules without the transactivation domain – a functional dominant/negative variant (Moriggl *et al.* 1996; Mui *et al.* 1996). Obviously, C-terminally truncated forms of Stat5 would not be immunoprecipitated by an antibody directed to the C-terminal portion of the Stat5 molecule. Furthermore, the specific results of the various Stat5 antibody immunoprecipitations on *Ssp*I digested plasmid were similar for experiments performed with the double digestion using endonucleases *Nde*Iand *Xba*I (data not shown), indicating the N-terminal antibody is the best available antibody for immunoprecipitation of Stat5-bound DNA elements.

Taken collectively, these initial results provided a strong experimental basis for the development of a method to specifically identify Stat5 binding sites within the human genome. The establishment and optimization of the method using a Stat5 responsive promoter in an *in vitro* and cell-free setting provided the preliminary data needed for transferring this technology to whole cells or tissues. The ability to eliminate many variables and optimize the antibody concentration and immunoprecipitation conditions was a very beneficial and necessary first step in the development of new methodology for identifying Stat5 binding sites within the human genome.

Method Application and Development in a Cell Culture Setting

In the next critical step in the development of a method for genome-wide identification of specific Stat5-chromatin interaction sites, T-47D human breast cancer cells were selected as an experimental model. These cells were chosen for several

reasons. First, T-47D cells are relatively well-differentiated with an epithelioid phenotype compared to other less differentiated, mesenchymal or fibroblast-like breast cancer cells. Second, T-47D cells have a relatively high level of protein expression of Stat5, PrlR, and Jak2 – all critical components for proper Stat5 signaling and function (Shiu 1979)(Rui, unpublished observations). In contrast, many of the less well-differentiated breast cancer cell lines have lost either the expression of Stat5 or the ability to signal through Stat5 because of alterations in the signaling machinery. Third, while the work presented in the first segment of the *Results* section was carried out on HC11 mouse mammary cells, T-47D cells were chosen because they are human and therefore most relevant for studies of human breast cancer. The availability of the human genome also made the selection of a human breast cancer model preferable, because newly identified Stat5 interaction sites could be quickly mapped to a specific sequence of the genome.

Adenoviral Gene Delivery of Stat5 Variant with Enhanced DNA Binding

In order to maximize the effectiveness of the efforts in establishing this method, several optimization steps were added. First, it was hypothesized that the overall level of Stat5 expression, activation, and association with DNA would affect the effectiveness of capturing Stat5 bound to chromatin. To maximize binding of Stat5 to available DNA interaction sites in the T-47D cells, the author elected to overexpress a variant of Stat5 with enhanced DNA binding capacity to achieve saturation of all possible Stat5 response elements. As previously mentioned, work performed in Dr. Hallgeir Rui's laboratory has generated an adenovirus for effective delivery of this Stat5 variant, *Stat5*Δ713.

Stat5Δ713 is able to bind DNA by EMSA (Yamashita *et al.* 2001). The molecule is missing the C-terminal transactivation domain, and acts as a dominant-negative as it is unable to promote the transcription of Stat5 regulated genes (Ahonen *et al.* 2003). Furthermore, work published in Dr. Rui's laboratory has shown that this molecule, when activated and dimerized, binds DNA-containing Stat5 consensus sequences tighter and for a longer duration when compared to the wild type protein. Therefore, *Stat5*Δ713 was selected for adenoviral delivery into T-47D cells to maximize the effectiveness of Stat5 binding and facilitate the method development.

Protocol Derivation and Application

Confluent cultures of T-47D cells in 100 mm dishes were infected with the adv-Δ713-*Stat5* at an MOI of 5. Conditions of the infection were as described in the *Materials and Methods* section and the cells were given serum-free medium. After 24 hours half of the dishes were treated with 10 nM human prolactin for 30 minutes while the others remained untreated.

The following protocol for the isolation and purification of Stat5-bound genomic elements is a combination of the previous cell-free Stat5-plasmid experiments and other established methods used to generate a library of cloneable Stat5-bound DNA fragments. It should be noted that this section details the development of the method including several steps that have since been improved. The most current protocol is listed in the *Materials and Methods* section.

After activation of Stat5 with prolactin, the cells were treated with 37% formaldehyde to a final concentration of 1% by the addition of 270 µl directly to the 10

ml of medium. The formaldehyde was left on the cells for a total of 45 minutes at 37°C. The dishes were covered and sealed in order to eliminate contamination to other cells in the incubator. Next, the medium was aspirated and the cells were washed with 5 ml wash/scraping buffer containing inhibitors (1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin A in ice cold PBS). The liquid was aspirated and 5 ml additional ice-cold buffer was added and the cells were scraped and collected in a 15 ml conical tube. The cell solution was pelleted for 4 minutes at 1000 RPM at 4°C and the supernatant discarded. Unused cell pellets were frozen and stored at -70°C while the other cell pellets were resuspended in 2 ml SDS-lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0) in water). All samples were then incubated for 10 minutes on ice.

The cell lysates were sonicated with a tapered microtip and the sonicator (Branson Sonifier 250, Branson Ultrasonics Corporation, Danbury, CT) was set to 50% power (maximum for microtip), continuous program, and tuned to minimum frequency. The samples were sonicated for 30 seconds total (3 repetitions of 10 seconds each). Samples were cooled on a dry ice/ethanol bath between sonication cycles and stored on wet ice when finished. All samples were then centrifuged at 13,000 RPM for 10 minutes at 4°C to precipitate the solid cellular lysate components and the supernatant was transferred to a fresh tube.

Next the samples were diluted 10 fold in immunoprecipitation (IP) dilution buffer (0.1% SDS, 1.1% Triton X, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 16.7 mM NaCl, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). After dilution, 1% of the total sample (~50μl) was removed for an analysis of sonication effectiveness by running a portion of the sample on an ethidium bromide stained agarose gel. The remaining sample

was then pre-cleared by the addition pre-washed protein A-Sepharose beads (50% protein A Sepharose bead slurry, 20 µg sonicated salmon sperm DNA, 0.1% BSA, in TE (pH 8.0) for 30 minutes at 4° with rotation. The beads were then pelleted by centrifugation at 700 x *g* for 5 minutes, supernatant was collected and transferred to a fresh tube. Each sample's supernatant was split into 2 equal portions for parallel immunoprecipitations using the previously determined and optimized N-terminal anti-Stat5 antibody and the NRS negative IP control. Each of the samples was given 1 µg of the respective antibody and was incubated with rotation for 2 hours at 4°C.

The immune complexes were collected by the addition of 60 µl pre-washed beads for 1 hour at 4°C. The beads were pelleted by centrifugation for 5 minutes and at this point immune complexes may be frozen at -70°C, if necessary. Next, the beads were washed with 5 buffers, 3 times for each buffer, adding 1 ml per wash and incubated for 3-5 minutes. The following is the composition of each buffer: wash buffer A (0.1% SDS, 1% Triton X, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl); wash buffer B (0.1% SDS, 1% Triton X, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl); wash buffer C (1% NP-40, 1% Sodium Deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1), 0.25 M LiCl); wash buffer D (TE, pH 8.0); wash buffer E (TE, pH 8.0).

The immune complexes were then chemically eluted from the beads by adding 250 µl of elution buffer (1% SDS in 0.1 M $NaHCO_3$). The sample was vortexed, incubated for 15 minutes at room temperature with rotation, and centrifuged to pellet beads. The supernatant was transferred to a fresh tube and the elution was repeated in 250 µl additional elution buffer, vortexed, and incubated for 15 minutes at room temperature. The eluates were combined for a total volume of 500 µl. Next, the

formaldehyde cross-links that covalently cross-linked the activated Stat5 to the respective genomic element were reversed by the addition of 20 µl of 5 M NaCl for a final concentration of 0.2 M and incubated at 65°C for 4 hours. After that, the proteins were digested by the addition of 10 µl of 0.5 M EDTA, 20 µl of 1 M Tris-HCl (pH 7.6), and 2 µl 10 mg/ml Proteinase K at 45°C for 1 hour.

The immunoprecipitated DNA was then recovered by phenol:chloroform extraction and ethanol precipitation and the final product was resuspended in 30 µl of TE, pH 8.0. Because of the relatively small size and amount of the sonicated DNA fragments, 20 µg of glycogen was added as a carrier in the ethanol precipitation step.

Quantification of the Enriched Pool of Stat5-Bound Elements

Several independent analyses were made in an attempt to quantify the amount of DNA present in the final product from the previously described protocol. Agarose gel electrophoresis was attempted and determined to be not sensitive enough for visualization of ethidium bromide stained samples (data not shown). Additionally, a set of serial dilutions of the final product was established in an attempt to use an UV spectrophotometery to quantitate the amount of final product present (data not shown). The final analysis was unable to reliably determine the amount of recovered product, probably due to the limits of detection of the UV spectrophotometer.

In a separate attempt to characterize the amount of product recovered in the final enriched pool of prospective Stat5 bound genomic response elements, an ethidium bromide spot test was used. Briefly, serial dilutions of DNA were created at known concentrations and then were mixed with ethidium bromide and dotted on a

polycarbonate plate and visualized under an ultraviolet light. These dilutions were then compared with the samples of interest including a portion of the pre-phenol:chloroform extraction product, as well as the final purified and precipitated product. In serial dilutions of DNA stocks ranging from 20 μg/ml to 10 ng/ml, no detectable DNA was observed in the enriched pool (data not shown), indicating that the levels of DNA present in the final pool were below the sensitivity of ethidium bromide staining and UV visualization with the imaging equipment available (Stratagene Eagle Eye, La Jolla CA). Although the amount of Stat5-bound DNA fragments were below the limit of detection by these methods when using the amount of starting cell material as described, the purified DNA fragment pool could be further processed and a series of cloning techniques were used to identify immunoprecipitated DNA elements, as described later.

Sonication Optimization

Another important step in the development of this method was to optimize the conditions for sonication of the cell pellets. Sonication is necessary to shear the genomic DNA into fragments that could be cloned and sequenced, but remain large enough to specifically identify the exact region of the genome from which the fragment originated. It was determined that approximately 400 base pair (bp) fragments would give the best possible compromise of specificity and workability. Specifically, 400 bp fragments allow for a complete sequencing read-through and is more than sufficient to localize the fragment within the human genome with a high degree of statistical certainty.

As previously noted, the volume to be sonicated was optimized to be 400 μl in a 1.5 ml microcentrifuge tube. This provides enough volume to completely immerse the

sonicator microtip in the cell pellet/lysis buffer mixture, which decreases the possibility for sample foaming and inhibiting fragmentation while not allowing the microtip to contact the inner tube surface.

The following experiment was performed on T-47D breast cancer cell pellets, taken from 175 cm^2 flasks and resuspended in 400 µl of the previously mentioned lysis buffer. The sonicator used for these experiments was a Fisher Scientific Sonic Dismembrator™, model number 500 (manufactured by Branson Ultrasonics Corporation, Danbury, CT), fitted with a tapered microtip and was set with an amplitude of 50%. After sonication, the lysate was diluted 10 fold in PBS (to be equivalent to the dilution with immunoprecipitation buffer in the cloning assay), and 15 µl was applied to a 2% TBE-agarose gel stained with ethidium bromide. As shown in the Figure 23, there is substantial variation in the effectiveness of DNA fragmentation based on time of sonication. Intuitively, the length of sonication is inversely proportional to the length of DNA fragments, e.g. the more energy put into the system the greater DNA disruption. However, it is interesting to note that the total amount of time is not the determining factor for the end product size. As can be seen in Figure 23, 60 seconds of sonication in 2 bursts of 30 seconds each was much more effective in shortening the average length of DNA fragments compared to 4 bursts of 15 seconds each. In the sample with the shorter sonication times, it should also be noted that while the predominant species remains the larger fragment size, a small portion are degraded to the optimal size.

The 4 samples Figure 24 are from a representative experiment performed on T-47D human breast cancer cells treated with or without prolactin. The samples are taken from the pre-immunoprecipitation stage and represent a portion of the total chromatin

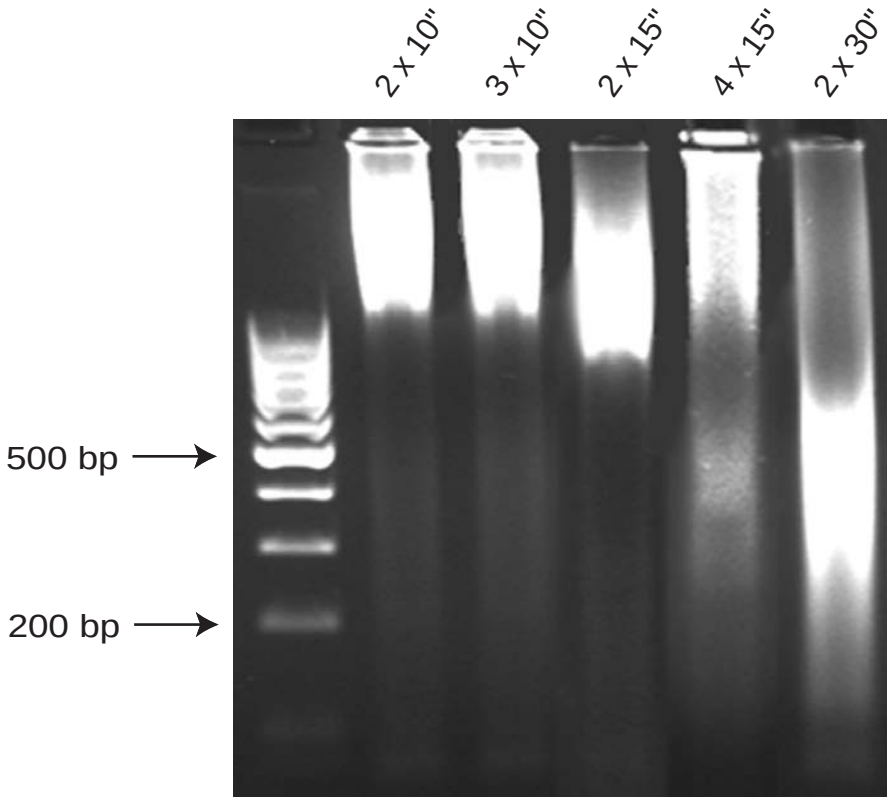


Figure 23. Optimization of sonication parameters for human T47D breast cancer cells. After sonication a portion of the samples were run on an agarose gel stained with ethidium bromide and visualized by UV light. All samples were sonicated in 400 µl of lysis buffer in a microcentrifuge tube under the sonication conditions described in the text. The number and time of each sonication pulse is listed above each sample.

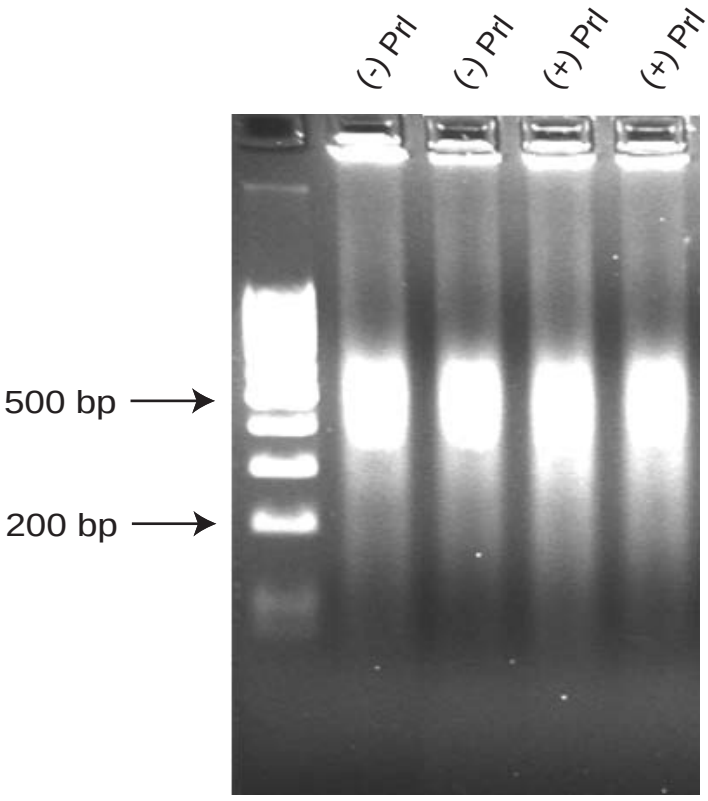


Figure 24. Sonication of human genomic DNA resulted in average fragment size of approximately 400 base pairs. T47D human breast cancer cells were treated with or without Prl for 30 minutes before harvest. Cells were sonicated and pre-IP samples were run on an ethidium bromide stained agarose gel.

used. Note the relatively equal chromatin loading and equivalent chromatin fragmentation to the desired ~400 bp range.

Cloning of Stat5 Binding Sites for Further Characterization

Several alternate strategies were undertaken in order to most efficiently clone the pool of enriched Stat5 response elements into bacterial vectors for further characterization. Since sonication of the genomic DNA will result in a mixture of DNAfragments, including 5' and 3' overhangs of varying lengths (Elsner and Lindblad 1989), as well as blunted fragments, several alternatives for manipulation were available.

As a first option, *Taq* DNA polymerase was used to add a 3' Adenosine nucleotide to each double stranded DNA fragment. The 15 µl reaction was composed of 5 µl of immunoprecipitated sample, 1.5 µl of 10X PCR reaction buffer, 1 µl 25 mM $MgCl_2$, 4 µl 10 mM dNTPs, 0.3 µl *Taq* polymerase, and 3.2 µl H_2O. The mixture was incubated for 30 minutes at 72°C. After completion the fresh product was immediately ligated into a pCR 2.1 vector, using an Invitrogen TA cloning kit as per the manufacturer's recommendations. After a 24-hour ligation period the vector was transformed into TOP10 bacteria, per manufacturer's directions.

The bacteria were then plated on LB/agar plates with ampicillin and S-gal (Sigma, St. Louis, MO) for selection overnight at 37°C. Positive clones (white color) were then picked and grown overnight (16 hours) in 5 ml LB with ampicillin at 37°C and shaken at 225 RPM. Bacterial mini-preps were performed to isolate the transfected plasmid with the Wizard Plus SV miniprep kit (Promega Corporation, Madison, WI) following the manufacturer's protocol. The purified plasmid was used for primer extension dideoxy

sequencing in a standard protocol (4.0 µl reaction premix, 3.0 µl template, 1.0 µl 3.2 pmol/µl M13 Reverse Primer, and 12 µl dH_2O for a 20 µl reaction) under the following conditions: 96° for 1 minute and then 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for 4 minutes. After cycling the final products were then purified by using an Amersham-Pharmacia (Piscataway, NJ) G-50 column.

The sequence analysis showed no significant inserts present in any of the clones selected (data not shown). Since it was known that only a fraction of samples would have been available for TA cloning due to the random nature of DNA fragmentation by sonication, an alternative cloning technique was tested. The improved protocol increased the number of available fragments for cloning, thus improving the overall efficiency of the technique.

T4 DNA polymerase was selected to modify all DNA fragments since it has the ability to fill-in 3' overhangs (5' to 3' polymerase) and remove 5' overhangs (3' to 5' exonuclease), thus forming blunt ends on all fragments. The reaction was carried out under standard conditions (5 µl of immunoprecipitated product, 2 µl 10X reaction buffer [working concentrations: 3.3 mM Tris-acetate, 6.6 mM K-acetate, 1.0 mM Mg-acetate, and 0.01 mg/ml BSA], 10 µl 1.0 mM DTT, 1.6 µl 10 mM dNTP, and 1.4 µl dH_2O) and incubated at 37°C for 5 minutes. The sample was diluted with water to 200 µl following the reaction. The blunted fragments were then recovered by phenol:chloroform extraction followed by ethanol precipitation and then resuspended in 30 µl dH_2O.

A single 3' Adenosine residue was added by *Taq* polymerase as previously described for ligation into a TA cloning vector and the bacteria were plated on ampicillin- and S-gal-containing selection plates. Purified plasmid samples were digested and run on

an agarose gel to identify and qualify the presence of inserts. Positive clones with visible inserts were then sequenced as previously described. Although the described blunt end cloning strategy gave a higher frequency of positive clones (by blue/white colony screening), the quality of insert determined by sequencing was not satisfactory, so further improvement of technique was needed.

In order to increase the cloning efficiency, electrocompetent *E. coli,* DH-5αE (Invitrogen, Carlsbad, CA) were used. The manufacturer's recommendations were followed for the transformation in a Bio-Rad Gene-Pulser (Hercules, CA), except 50 µl of cells and 2 µl of ligated vector were used.

At this point the overall method was streamlined and cost was significantly reduced by eliminating the overnight amplification of the individual colonies, the plasmid mini-prep purification, and the endonuclease digestion. PCR amplification of inserts could be performed directly on the bacterial colonies by picking each colony with a sterile pipette tip, then adding each sample to a separate tube containing the PCR reaction mixture on ice. PCR primers were used that flanked the TA cloning site in the pCR 2.1 vector; specifically, the M13 reverse primer is located 5' to the site and T7 primer is on the complementary strand and downstream of the cloning site. A 25 µl PCR reaction was set up under standard conditions (2.5 µl 10X PCR buffer, 1.75 µl 25 mM $MgCl_2$, 2.0 µl 10 mM dNTP, 0.15 µl *Taq*, 1.0 µl 3.2 pmol/µl M13 RP, 1.0 µl 3.2 pmol/µl T7 P, and 16.6 µl dH_2O) and was incubated at 94°C for 2 minutes to lyse the bacteria and inactivate any endogenous nucleases. The reaction was then cycled 36 times at 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 30 seconds and the products were run on an ethidium bromide stained agarose gel and visualized with UV light. In total, 58 white

colonies were picked and amplified, but showed no insert versus the control, no-insert, blue colony, as a representative sample shows in Figure 25.

Linker-Mediated PCR Amplified Cloning

Since the quantity of immunoprecipitated product in the final, enriched pool of Stat5-bound genomic response elements appeared to be below a critically low level for cloning, an alternate method was devised to attempt to increase the population ofcloneable elements. A unidirectional linker was added to each end of the blunted fragment, which provided a template to specifically amplify the elements and provide a better ratio of fragment to vector in the subsequent ligation reaction. As originally described in Ren, *et al* (Ren *et al.* 2000), 2 individual oligonucleotides were synthesized and annealed, and then ligated to the blunted fragments. Briefly, LINKER A was synthesized with the sequence: 5'-GCG GTG ACC CGG GAG ATC TGA ATT C - 3' and LINKER B was synthesized with the sequence: 5'- GAA TTC AGA TC - 3'. The 2 oligonucleotides were then annealed under standard conditions (Ausubel *et al.* 1992) by adding 20 μM of each linker in 250 mM Tris-HCl (pH 7.7). The mixture was heated to 95°C for 5 minutes, then controllably cooled to 22°C over 1 hour, left at 22°C for 1 hour, subsequently progressively cooled to 4°C over 1 hour, then left at 4°C for 12 hours, and finally transferred to -20°C for storage. It is important to note that during the synthesis of the oligonucleotides, the termini are not phosphorylated, and therefore will not ligate end-to-end. It should also be noted that LINKER B is complementary to 3' end of LINKER A, therefore, when annealed the molecule will have a blunt end on one end and a 5'

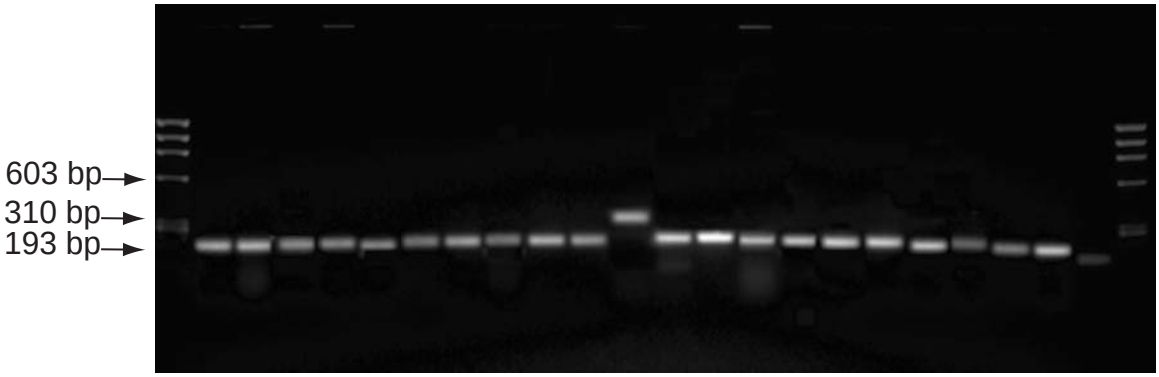


Figure 25. Representative bacterial colony screen with no significant inserts present. As described in the text, no inserts were present in colonies as a result of this cloning method. All analysis was done on white colonies, suggesting poor cloning efficiency in this experiemental protocol.

overhang on the other (for a diagrammatic illustration see Figure 26). This is the basis for the unidirectional cloning when associated with a double stranded blunt product.

The blunted immunoprecipitated product was then ligated to the unidirectional linkers under standard T4 DNA ligase procedures. PCR was used to amplify the pool of fragments for better cloning efficiency. A standard 50 µl PCR reaction was used with LINKER A as the only primer. As illustrated in Figure 26, only one primer is needed since there is complementary sequence for the primer on each side of the insert. The mixture was amplified by cycling 35 times under standard conditions.

A portion of the products was applied to a gel to visualize the product size and estimate the quantity of product. A smear was present in the 400-700 bp range suggesting amplification of the cloned and linker-ligated fragments (Figure 27). The variation in size of products was presumably due to the random length of DNA fragments generated by sonication. To verify the specificity of the reaction, a portion of the products from the previous reaction were diluted and subjected to another amplification, using the same primer and conditions, with only 25 cycles. It was expected that the products would have increased in intensity in the smear, but remain at the same size range. However, the smear remained roughly the same intensity and increased to a size consistent with 1000-2000 bp (data not shown). The increase in size was attributed to excess linker complexes and consequent annealing with the PCR products with the 3' A overhang generated by *Taq* polymerase. This was verified by sequence analysis of clones generated using that product, which showed a repeating sequence from the linkers.

Figure 26. Illustration of unidirectional linker-mediated cloning strategy. As a first step, the immunoprecipiated Stat5 response elements (red) are uniformly blunt-ended to maximize cloning efficiency. Complementary oligionucleotides were first annealed, then ligated as a unidirectional linker (blue) to the product. PCR amplification with a low number of cycles increased the pool of clonable elements, while not drastically altering the ratios of products. The *Taq* polymerase used in PCR amplification also fills in (green) the 3' short end created by the linkers and generates the 3' A overhang necessary for TA cloning into the vector (black).

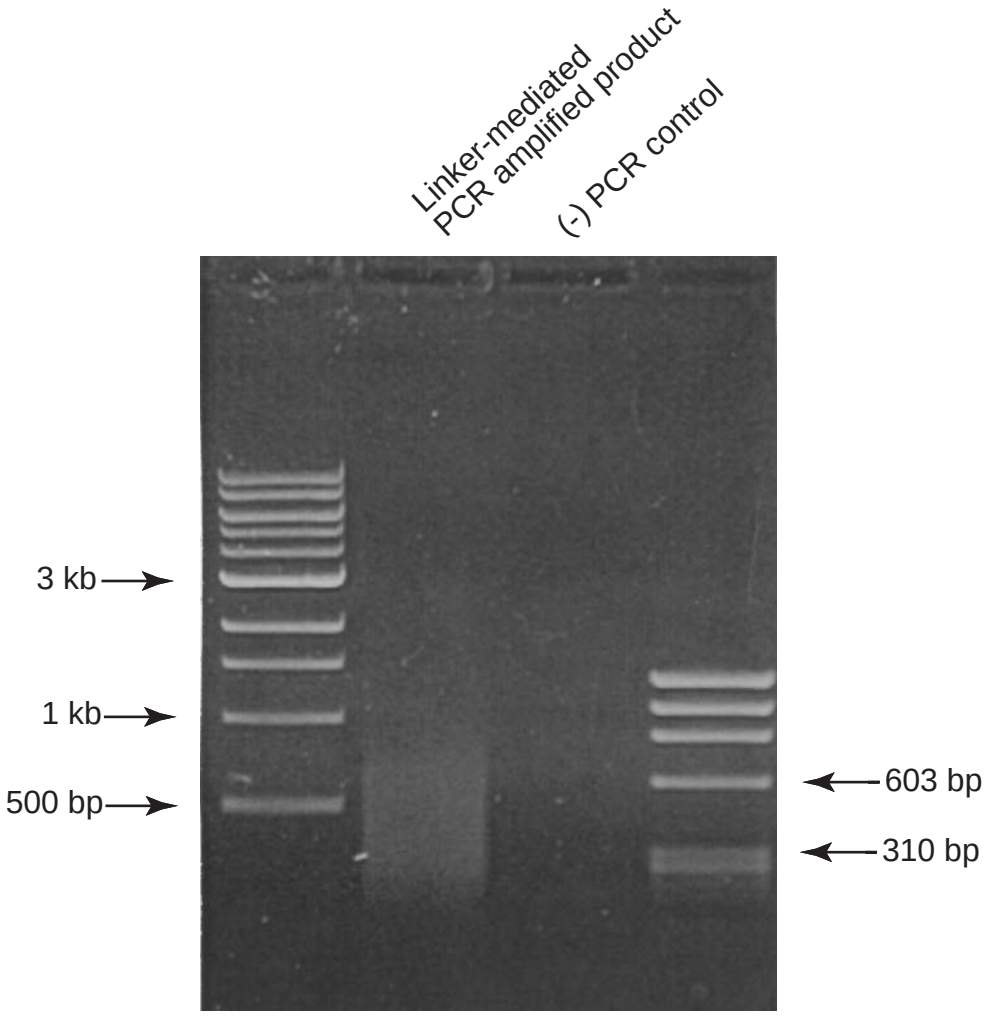


Figure 27. Linker-mediated PCR amplification of immunoprecipitated Stat5 genomic response elements. As described earlier, enriched Stat5 response elements were modified by adding a unidirectional linker to each side of the blunt-ended fragment. A low number of PCR cycles then increased the quantity of each fragment for future cloning and analysis. The negative PCR control contained all reaction components except template.

To alleviate the interference from the excess annealed linkers as well as T4 DNA ligase, the DNA was precipitated using a standard Na-acetate/ethanol protocol before amplification with PCR as described above. Again, the product from the initial amplification was diluted and amplified again to assure the specificity of the product. Under these conditions there was not an increase in DNA size and the product was specifically reamplified, suggesting that the fragments would be suitable for cloning (data not shown).

For the cloning of the linker-mediated amplified products, the following conditions and ratio was used: 7.0 µl of PCR product, 1.0 µl 10X ligase buffer, 1.0 µl pCR 2.1 TA cloning vector, and 1.0 µl T4 DNA ligase; and the mixture was incubated overnight at 14°C. The ligated vector was transformed as described previously by electroporation, and bacterial colonies (see representative colonies in Figure 28) were subjected to direct PCR amplification to identify positive colonies that contained inserts. A representative selection of PCR products of cloned Stat5-chromatin interaction sites, taken from positive (white) colonies is shown in Figure 29. PCR products of colonies that were selected for further analysis due to visible insert were first purified using a QIAquick™ PCR purification kit (Qiagen, Valencia, CA), followed by sequencing and analysis, as discussed later.

Random Hexamer-Mediated PCR Cloning

Although the author had arrived at an effective cloning procedure, the author also explored the efficacy of yet another approach. As shown in prior experiments, cloning efficiency was greatly improved when using some amplification (6-10 cycles) of the

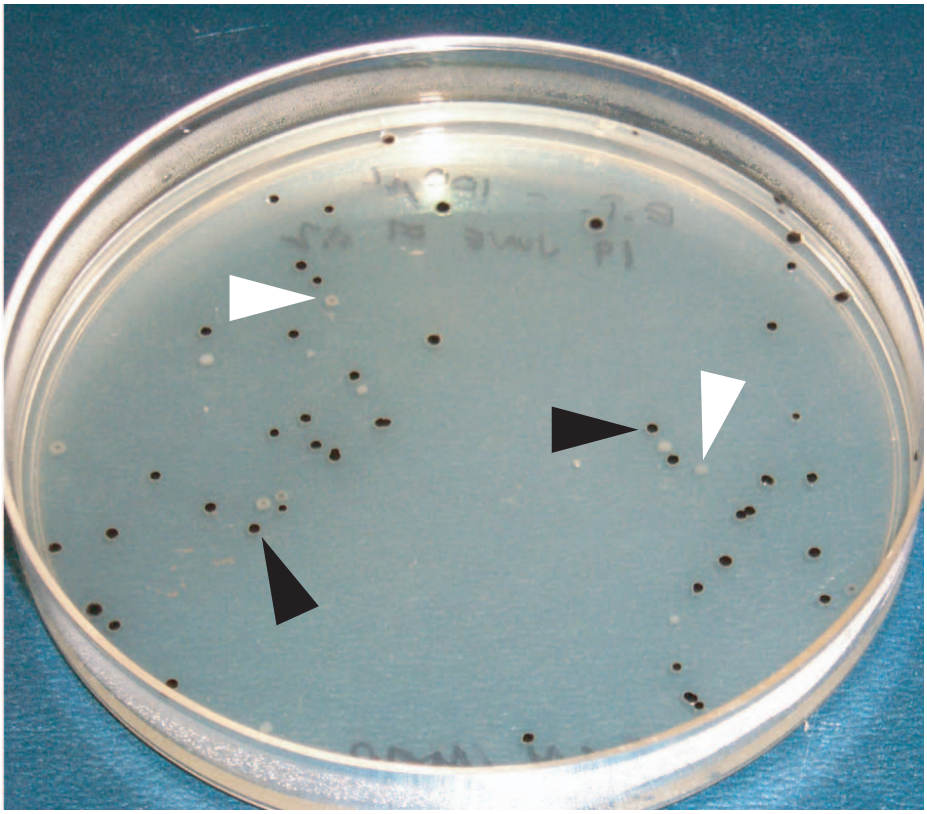


Figure 28. Representative bacterial colony distribution for cloned Stat5 genomic response elements. After transformation with antibiotic resistance containing plasmids, bacteria were plated on a S-gal based agar plate, as described in the text. White colonies (highlighted with white arrows) contain an insert and were analyzed further. Black (negative) colonies (highlighted with black arrows) are also shown and were used as a negative insert control in future PCR screening.

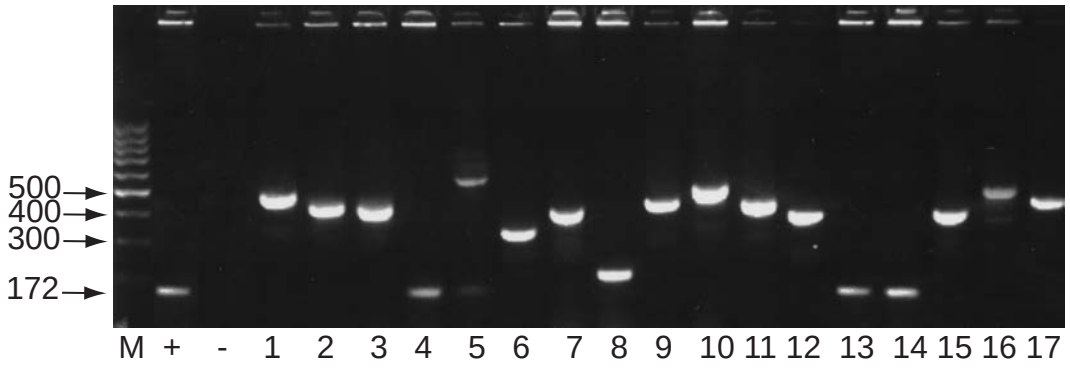


Figure 29. Representative cloned Stat5-bound chromatin fragments from prolactin-stimulated T47D breast cancer cells. PCR amplification of inserts from white colonies using primers flanking the cloning site. Samples were run on an ethidium bromide stained agarose gel and analyzed for the presence and size of insert. Marker is 1 Kb ladder (M); + denotes vector control (no insert); - denotes negative control. Note presence of insert in the majority of white colonies.

library, presumably by increasing the insert-to-vector ratio. Because it is possible that the linker-mediated amplification of immunoprecipitated fragments would preferentially amplify the most abundant species, thus skewing the population of cloned elements, the author also explored another possibility for amplification an alternative method to achieve non-specific, and hence non-preferential, amplification of the initial DNA fragment pool.

A pool of random hexamer primers was added to the final enriched pool of Stat5-bound genomic elements and PCR was used to amplify the pool. The 50 µl reaction was cycled 15 times at 94°C for 1 minute, 36°C for 1 minute, and 72°C for 2 minutes after an initial denaturation of 94°C for 2 minutes. The reaction was performed under standard conditions (10 µl template, 6.0 µl 293 pmol/µl random hexamer primer, 4.0 µl 10 nM dNTP, 3.5 µl 25 mM $MgCl_2$, 5.0 µl 10X PCR buffer, 0.5 µl *Taq*, and 21 µl dH_2O). The products were run on an agarose gel but no product could be detected with UV illumination (data not shown). Nonetheless, an attempt was made to clone the randomly amplified product.

In total, 131 positive colonies were picked and analyzed for an insert by running the products of the PCR amplification on an agarose gel. None of the PCR products from this technique of cloning showed an insert present when compared to the positive PCR (no insert) control (data not shown). It is possible the lack of product was due to an initial problem with the DNA pool after the random amplification of the immunoprecipitated fragments. It also possible that a lack of cloneable inserts was due to a problem with the functionality of the fragments after the hybridization with the random

hexamers, since a large proportion may have been amplified as very short fragments and could have significantly disrupted the ratio for optimal large fragment cloning.

Cloning Technique Summary

Several independent strategies were tested for cloning of the immunoprecipitated Stat5-chromatin interaction sites. While in theory each strategy provided specific benefits and possible improvements, the limitations with the given cloneable pool provided variable results. In our hands, and in the work limited to this dissertation, the linker-mediated cloning technique provided the best overall cloning-to-sequencing results. The use of the linker-mediated amplification and unidirectional linkers for high-efficiency cloning has yielded excellent sequence quality from the cloned inserts.

Sequence Analysis

After having successfully optimized the cloning strategy of captured Stat5-bound DNA fragments, coupled with successful direct sequencing from bacterial colonies without the need for minipreps, the sequence of individual inserts were mapped to a location in the human genome. The following describes the method used for the sequence analysis of clones. The raw sequencing data were compared to the sequence of the parental vector using BLAST2 (Basic Local Alignment Search Tool) on the National Institutes of Health, National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) for a pairwise comparison. This was used to delineate the origin and terminus of the cloning vector within the overall sequence of the sample. The sequence of the unidirectional linker was then removed.

Once determined, the sequence of the actual cloned insert, minus the linkers and vector, was subjected to a human genome specific BLAST search. Default parameters in the algorithms were used initially for all searches: *Program = blastn*, *Expect = 0.01*, and *Filter = default*. The initial results give localization to the general region of the human genome, including the specific chromosome and arm. Further analysis revealed the precise location of the hit and included the relationship of the specific hit to the strand and orientation of the neighboring genes.

In cases of sequences with regions of repetitive nucleotides or when the sequenced insert did not match a region within the human genome throughout the length of the insert, the parameters for BLAST analysis were altered. For repetitive sequences, or sequences that contained localized regions of repetitive sequence, the filter was changed from *default* to *none* to decrease the stringency determined by the algorithm. In addition, especially for shorter sequences that were not determined to be statistically significant, the expect value was changed from 0.01 to 1. These changes allowed an increased number of clones to be analyzed and localized within the human genome while also allowing a statistical threshold to verify the genomic location of each site.

Table 4 lists a representative sample of candidate Stat5-regulated genes, determined by the technology described here for the genome-wide identification of Stat5-chromatin interaction sites. The genes were identified by their proximity to the cloned and sequenced Stat5-genomic DNA interaction sites.

Table 4. Representative Sample of Candidate Stat5-Regulated Genes Based on Cloned Stat5-Chromatin Interaction Sites

FLJ10462: hypothetical protein; **FLJ11088**: hypothetical protein; **LOC51290**: CDA14; **PTHLH**: parathyroid hormone-like hormone; **UBASH3A**: ubiquitin associated and SH3 domain containing protein; **ZNF295**: zinc finger protein 295, **TFF2**: trefoil factor 2 (spasmolytic protein 1); **TFF3**: trefoil factor 3 (intestinal); **HAGE**: DEAD-box protein; **MGC10818**: hypothetical protein; **RPL39**: ribosomal protein L39; **CGI-02**: Highly similar to *S. cerevisiae* Mto1; **FLJ20548**: hypothetical protein; **PRX2**: paired related homeobox protein; **AD-003**: gene with protein product, function unknown; **PTGES**: prostaglandin E synthase; **FLJ22995**: hypothetical protein; **FLJ11736**: hypothetical protein; **RPS20**: ribosomal protein S20; **MOS**: v-mos Moloney murine sarcoma viral oncogene homolog; **ERCC6**: excision repair cross-complementing rodent repair deficiency, complementation group 6; **BMI1**: murine leukemia viral (bmi-1) oncogene homolog; **FLJ10851**: hypothetical protein - Highly similar to OGDH (2-oxoglutarate dehydrogenase); **CHAT**: choline acetyltransferase; **APM2**: adipose specific 2.	**CDKN3**: cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase); **TC10**: ras-like protein; **NIN**: ninein (GSK3B interacting protein); **PYGL**: phosphorylase, glycogen; **NLVCF**: nuclear localization signal deleted in velocardiofacial syndrome; **CLTCL1**: clathrin, heavy polypeptide-like 1; **UFD1L**: ubiquitin fusion degradation 1-like protein; **CDC45L**: CDC45 (cell division cycle 45, *S.cerevisiae*, homolog)-like protein; **CLIC2**: chloride intracellular channel 2; **FLJ10727**: hypothetical protein; **FLJ10975**: hypothetical protein; **IL9R**: interleukin 9 receptor; **MSX1**: msh (*Drosophila*) homeo box homolog 1 (formerly homeo box 7); **BST1**: bone marrow stromal cell antigen 1; **FLJ10297**: hypothetical protein; **ZNF141**: zinc finger protein 141 (clone pHZ-44) - C2H2 zinc-finger protein 141; **KIAA0716**: hypothetical protein; **DKFZP586B2022**: testin; **IFRD1**: interferon-related developmental regulator 1; **FLJ13576**: hypothetical protein; **FLJ21625**: hypothetical protein; **FLJ13465**: hypothetical protein; **BMPR1A**: bone morphogenetic protein receptor, type IA;

Validation Using Known Binding Sites/Genes

As an initial validation of the specificity of the new methodology for identification of binding sites for Stat5 within the human genome, an approach was taken that utilizes previously known characteristics of Stat5-responsive genes. In particular, the author took advantage of earlier work that has identified a group of Stat5 regulated genes that have been shown to contain the Stat5 consensus sequence, TTCNNNGAA. Using this information, oligonucleotide primers were designed that flanked the specific binding site for Stat5 within the promoter of the respective Stat5 responsive genes, then were amplified by PCR.

The protocol was followed in essence as previously described for the purification of Stat5 binding sites within the human genome and performed in T-47D human breast cancer cells. After immunoprecipitation, the enriched pool of Stat5 bound elements was phenol:chloroform extracted and precipitated overnight in ethanol at –20°C. The following day the DNA was resuspended in 30 µl of dH_2O.

Because previous experiments had shown that when using the established conditions for sonication, genomic DNA is fragmented to an average size of 400 bp, the author determined the optimal size for the validation PCR products to be 200 – 300 bp. PCR was performed using specific primer pairs on pools of enriched Stat5 binding sites derived from cells treated with or without prolactin, then immunoprecipitated with specific anti-Stat5 antibodies or with non-specific, purified IgG.

As illustrated in the following group of figures (Figures 30-35), the method provided a very powerful technique to analyze the status of Stat5 within the cell. Specifically, in the case for *β-Casein* milk protein gene Stat5 is induced to associate with

the promoter after treatment with prolactin, but not without stimulation (Figure 30). Specificity for the immunoprecipitation was shown by lack of an amplified product in the lanes with a non-specific IgG immunoprecipitation, regardless of prolactin stimulation. Therefore, the method of immunoprecipitating Stat5 responsive elements was validated, because Stat5 specifically associated with the known Stat5 binding site in the gene promoter only after activation by prolactin, and not in the absence of activation.

Some biological implications can also be made with respect to Stat5 function within a mammary epithelial cell. In the previous experiment the data suggest a relationship of prolactin treatment and Stat5 activation with the pro-differentiation marker, β-casein, a milk protein gene.

Similarly, in a parallel experiment with an alternate set of primers directed to the promoter of *Oncostatin M* (*OSM)* (Figure 31), prolactin-inducible Stat5 association to this promoter also could also be detected. Although initially described as a malignant phenotype suppressor (hence the nomenclature), the functions attributed to *OSM* varyconsiderably between cell types and assays in the literature. One can speculate that the prolactin-induced association of Stat5 with the promoter is driving a pro-differentiated or mitosis arrested state by the transcription of this gene.

When primers designed to the promoter of the *CIS-1* gene were used for a parallel experiment, specific and inducible Stat5 association with the response element could be seen (Figure 32). Therefore, as with the previously mentioned known Stat5 responsive genes, prolactin is able to induce an association with the *CIS-1* promoter in T-47D cells. It should also be noted that in this figure amplification of genomic DNA was shown as an additional control (in addition to the pre-immunoprecipitation sample) to verify the

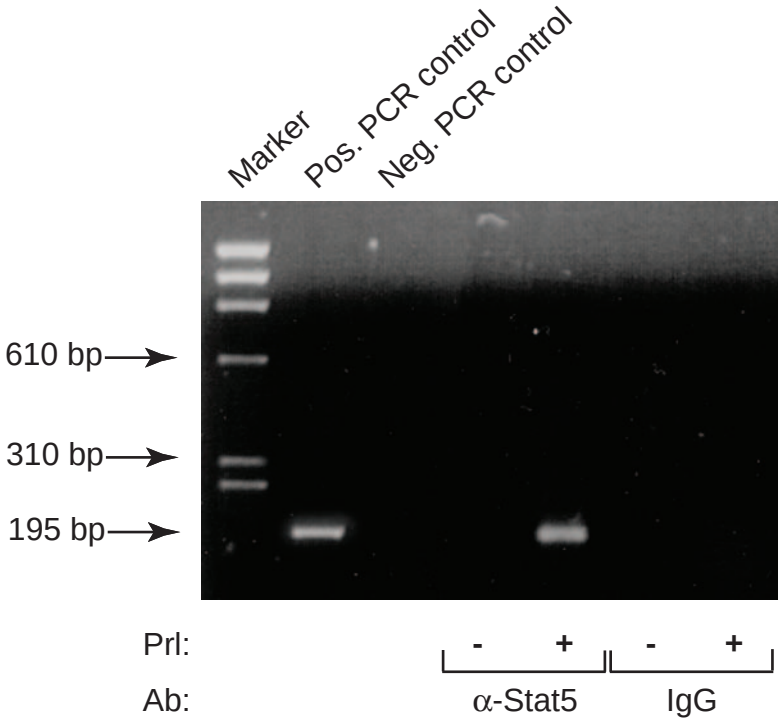


Figure 30. Prolactin activated Stat5 is able to specifically associate with the promoter of β-*Casein* in T47D human breast cancer cells. Cells were incubated with (+) or without (-) Prl for 30 min, then harvested as described. Non-specific IgG was used as a negative IP control and PCR was performed using primers designed to specifically amplify the promoters of the known Stat5-response element. Positive PCR control was performed using sonicated and purified Pre-IP sample for the template and negative PCR control was performed as a no-template reaction. Marker was ϕX174 DNA/*Hae*III digest.

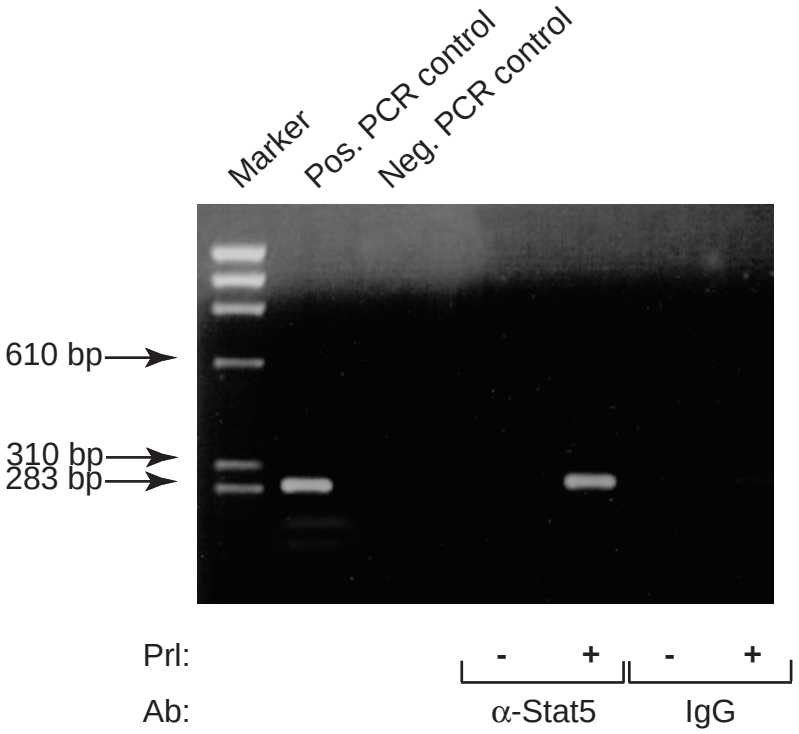


Figure 31. Prolactin activated Stat5 is able to specifically associate with the promoter of *OSM* in T47D human breast cancer cells. Cells were incubated with (+) or without (-) Prl for 30 min, then harvested as described. Non-specific IgG was used as a negative IP control and PCR was performed using primers designed to specifically amplify the promoters of the known Stat5-response element. Positive PCR control was performed using sonicated and purified Pre-IP sample for the template and negative PCR control was performed as a no-template reaction. Marker was ϕX174 DNA/*Hae*III digest.

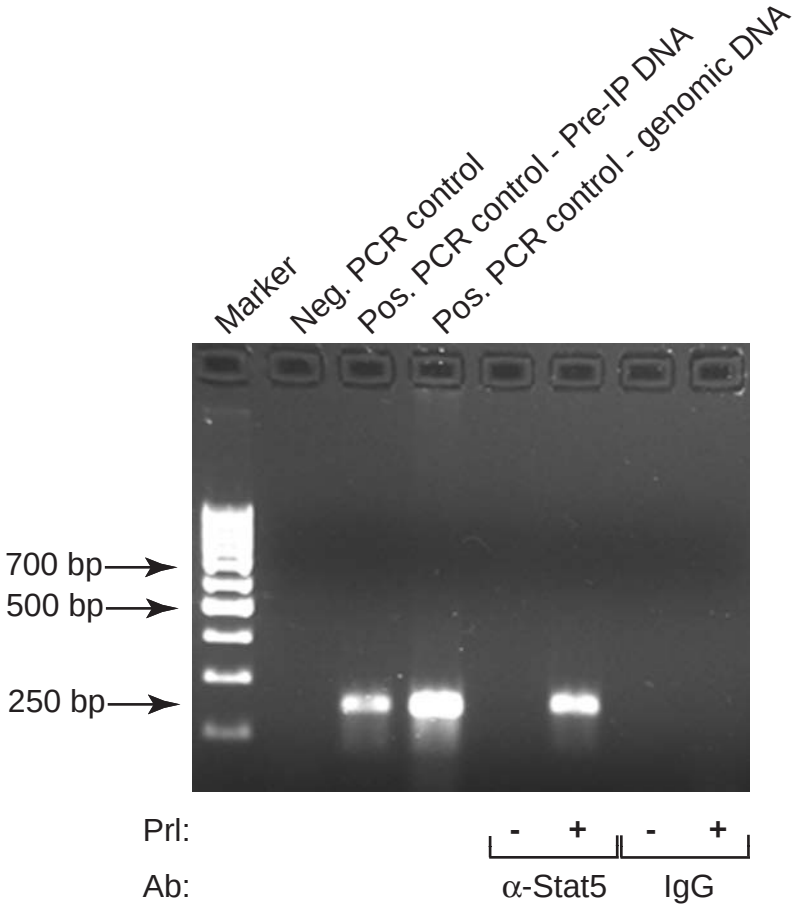


Figure 32. Prolactin activated Stat5 is able to specifically associate with the promoter of *CIS1* in T47D human breast cancer cells. Cells were incubated with (+) or without (-) Prl for 30 min, then harvested as described. Non-specific IgG was used as a negative IP control and PCR was performed using primers designed to specifically amplify the promoters of the known Stat5-response element. Positive PCR control was performed using sonicated and purified Pre-IP sample and purified genomic DNA for the template and negative PCR control was performed as a no-template reaction. Marker was 100 bp ladder.

specificity of the PCR amplification. This control reaction was performed for all primer sets, however was not incorporated in all other figures for simplicity.

Of equal importance is the recognition that simply being a Stat5 responsive gene in one setting (cell type, tissue type, in response to a given signal, etc.) does not necessarily mean Stat5 will associate with the promoter of that gene in all cell types and conditions. Specifically, *α2-macroglobulin* has been identified as a Stat5 responsive gene in liver, induced by growth hormone. As the results from the experiment in Figure 33 show, Stat5 is not able to associate with the promoter for this gene in T-47D breast cancer cells. Therefore, chromatin status and possibly the presence or absence of additional cofactors influence the ability of an activated transcription factor to bind to a specific genomic element.

In an experiment using the same pool of immunoprecipitated Stat5-bound elements as a PCR template and primers designed to the promoter of the *PrlR* (Figure 34) and *CDKN1A* – commonly referred to as $P21^{WAF1/CIP1}$- (Figure 35) similar results to *α2-macroglobulin* were seen. In T-47D under the culture conditions used, Stat5 did not associate with the *PrlR* or *CDKN1A* promoter, and the procedure was specific in that no product was detected in the pool immunoprecipitated with a negative control antiserum regardless of prolactin stimulation.

A parallel experiment was designed to detect Stat5 binding to the *Cyclin D1* promoter. A recent publication identified 2 distinct Stat5 consensus binding sites in the promoter for *Cyclin D1*, but only one could mediate transcription of the gene in a Stat5 dependent manner (Brockman, Schroeder, and Schuler 2002). Primers were designed flanking this site and a PCR reaction was carried out. Interestingly, in T-47D cells under

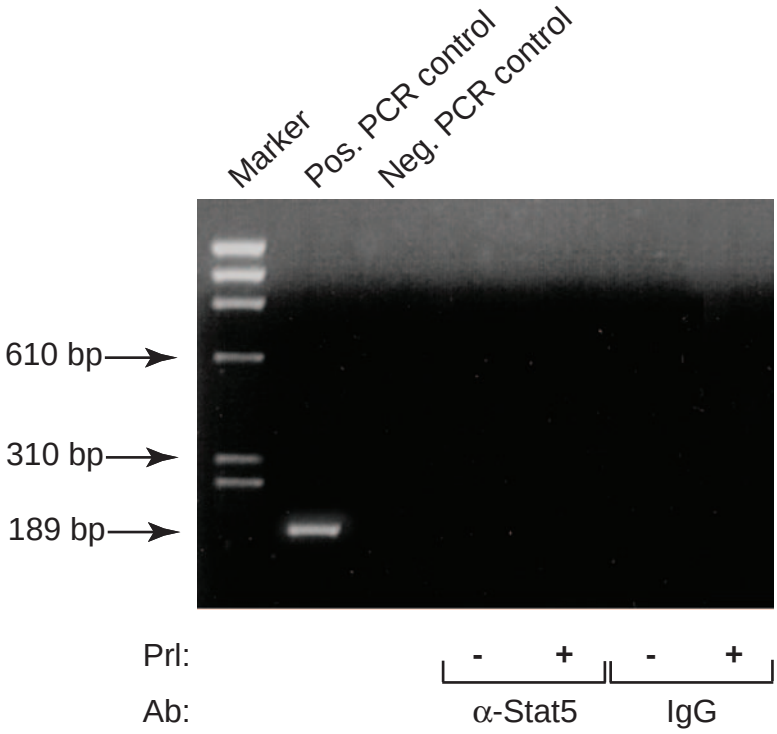


Figure 33. Prolactin activated Stat5 is not able to associate with the promoter of α*2-Macroglobulin* in T47D human breast cancer cells. Cells were incubated with (+) or without (-) Prl for 30 min, then harvested as described. Non-specific IgG was used as a negative IP control and PCR was performed using primers designed to specifically amplify the promoters of the known Stat5-response element. Positive PCR control was performed using sonicated and purified Pre-IP sample for the template and negative PCR control was performed as a no-template reaction. Marker was ϕX174 DNA/*Hae*III digest.

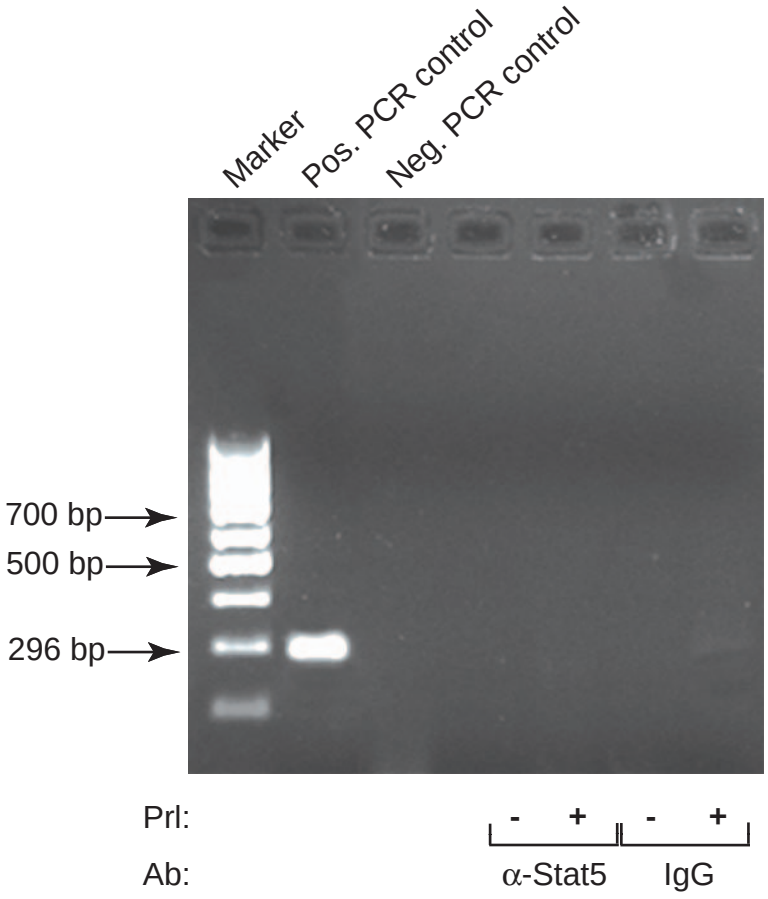


Figure 34. Prolactin activated Stat5 is not able to associate with the promoter of *PRLR* in T47D human breast cancer cells. Cells were incubated with (+) or without (-) Prl for 30 min, then harvested as described. Non-specific IgG was used as a negative IP control and PCR was performed using primers designed to specifically amplify the promoters of the known Stat5-response element. Positive PCR control was performed using sonicated and purified Pre-IP sample and purified genomic DNA for the template and negative PCR control was performed as a no-template reaction. Marker was 100 bp ladder.

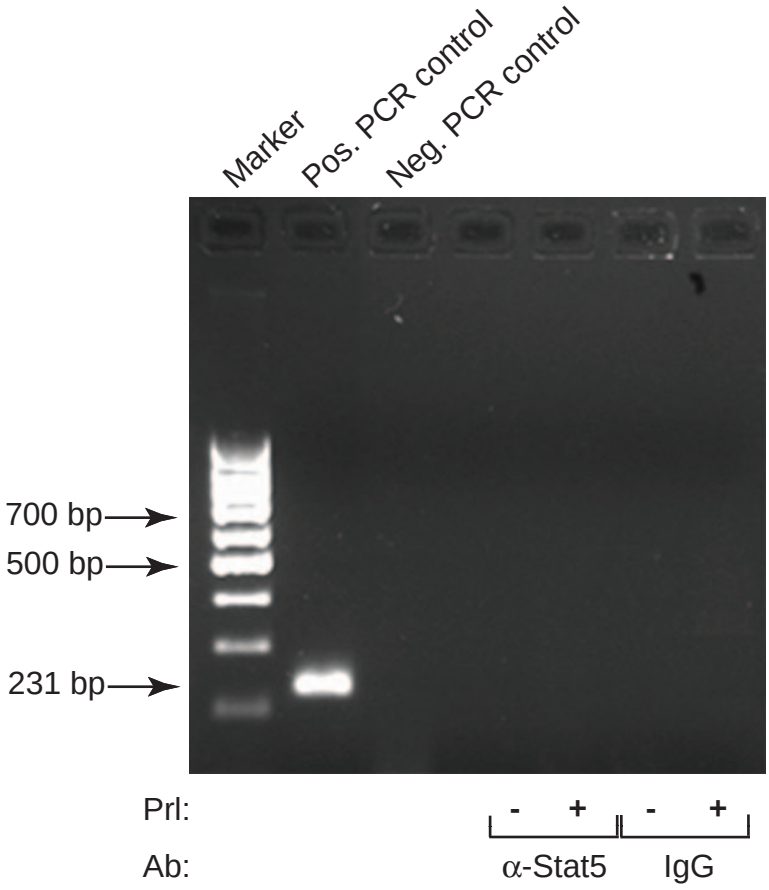


Figure 35. Prolactin activated Stat5 is not able to associate with the promoter of *CDKN1A* (*p21*$^{WAF1/CIP1}$) in T47D human breast cancer cells. Cells were incubated with (+) or without (-) Prl for 30 min, then harvested as described. Non-specific IgG was used as a negative IP control and PCR was performed using primers designed to specifically amplify the promoters of the known Stat5-response element. Positive PCR control was performed using sonicated and purified Pre-IP sample for the template and negative PCR control was performed as a no-template reaction. Marker was 100 bp ladder.

the conditions tested, Stat5 did not bind to the promoter of *Cyclin D1* after prolactin stimulation; however, during a starvation state without prolactin treatment Stat5 did associate with the promoter (Figure 36). The method of Stat5 activation and DNA binding to the *Cyclin D1* promoter is unclear. However, cross-talk between signaling pathways' or endogenous prolactin production and autocrine stimulation may sustain low levels of Stat5 activation that could be sufficient for *Cyclin D1* promoter association. It is possible that low levels of activated Stat5 bind to this promoter under serum starvation, but that prolactin stimulation leads to activation of additional factors that displace Stat5 from this promoter element.

In all of the previously mentioned experiments the positive PCR controls used the same primer sets as the experimental reactions, but the template was purified genomic DNA from T-47D cells (data not shown, except for Figure 32). The DNA was harvested using DNAzol™ reagent (Invitrogen, Carlsbad, CA) under standard conditions as described in the manufacturer's recommendations.

Additional PCR positive controls were also performed on the sonicated cell lysates removed before immunoprecipitation. These fractions were purified using phenol:chloroform extraction and ethanol precipitation before amplification to remove all cellular contaminants from the fragmented genomic DNA. This additional control verified that the primer sets were capable of PCR amplifying the specific region of sonicated DNA. This was necessary because if the sonication induced a consistent fracture of the DNA between the flanking primers, no amplifiable product would be visualized. In every case either method of positive PCR control provided similar results for all selected primers.

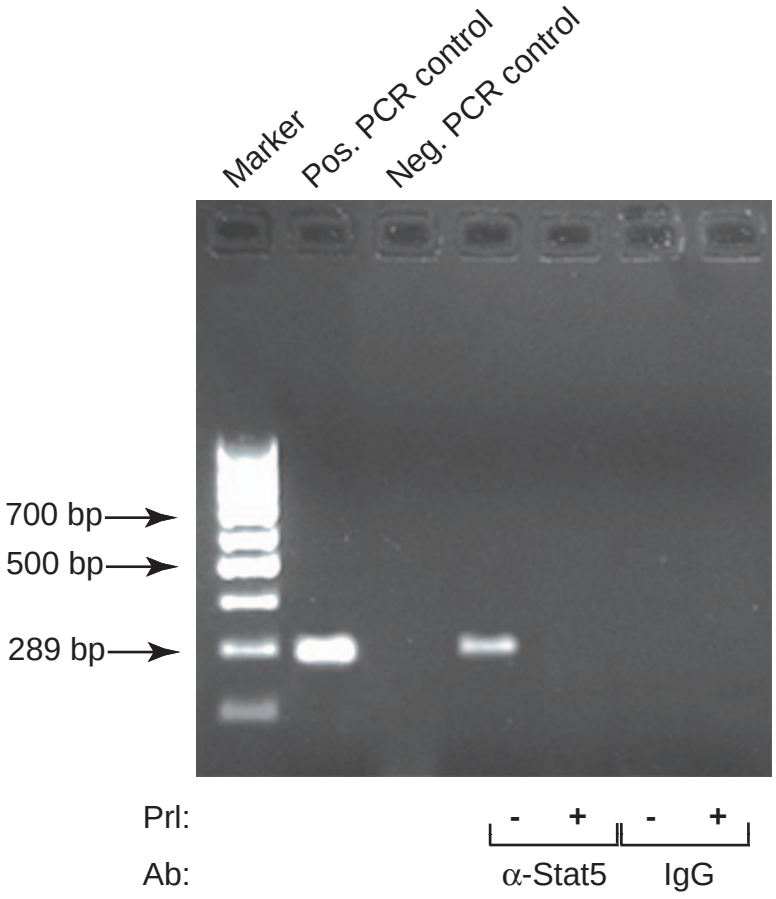


Figure 36. Prolactin activated Stat5 is not able to associate with the promoter of *Cyclin D1* in T47D human breast cancer cells, but does associate during a starvation state. Cells were incubated with (+) or without (-) Prl for 30 min, then harvested as described. Non-specific IgG was used as a negative IP control and PCR was performed using primers designed to specifically amplify the promoters of the known Stat5-response element. Positive PCR control was performed using sonicated and purified Pre-IP sample and purified genomic DNA for the template and negative PCR control was performed as a no-template reaction. Marker was 100 bp ladder.

Validation of Stat5 Binding to Cloned Novel Putative Stat5-Binding Chromatin Fragments – Use of Electrophoretic Mobility Shift Assay

An important step in establishing the new method for genome-wide cloning of Stat5-chromatin interaction sites was to establish a simple methodology for rapid validation of whether Stat5 indeed binds to the cloned sequence. The most specific approach to test this would be to examine whether activated, but not inactive, Stat5 forms a complex with the isolated fragment and that this complex of activated Stat5 can be specifically supershifted with antibodies to Stat5, but not with non-specific antibody.

Typically, EMSA is done using oligonucleotides of 20-40 base pairs. The cloned fragments to be tested in our analysis typically are between 200 and 400 bp, which required extensive optimization and modification of the EMSA protocol, as will be described. Our goal was to radioactively label individual PCR fragments and develop a highly sensitive method to detect Stat5 binding to the true Stat5-binding fragments. As described in detail in the *Materials and Methods* section, nuclear extracts from human T-47D cells were used (the same cell line from which the elements were cloned) after treatment with or without human prolactin. The author also explored whether adenoviral gene delivery of WT- or Δ713-*Stat5*, which displays enhanced binding, would increase the sensitivity of this assay. Increased sensitivity with less requirement for nuclear extract is important if the method is to be scaled up to screen a large number of clones.

EMSA of Cloned Stat5-Chromatin Interaction Sites

A complete experiment is shown in Figure 37 for DNA fragment #29. Free radiolabeled fragment was loaded in the first lane and verified the production of a single, specific product that was used in all the remaining lanes. Lanes 1-4 were loaded with the nuclear extract of T-47D cells treated without (lane 1) or with (lanes 2-4) 10 nM human prolactin to activate endogenous Stat5. No specific induction of Stat5 DNA-binding activity was detected in these cells in response to prolactin treatment with the amount of nuclear extract used. This could be overcome by increasing the amount of nuclear extract (data not shown), or by increasing the signal by expression of WT-Stat5 (lanes 5-8).

Lanes 5-8 of Figure 37 are parallel samples representing equal amount of nuclear extract from T-47D human breast cancer cells that have been infected with an adenoviral vector that contains WT-*Stat5*. After 24 hours of infection and starvation the cells were treated without (lane 5) or with (lanes 6-8) prolactin to activate Stat5 within the cells. Nuclear extracts were prepared and incubated with radiolabeled probe and specific Stat5 anti-serum or non-specific immunoglobulin, as indicated. As a result of prolactin stimulation a noticeable band can be seen demonstrating a specific, inducible DNA-binding complex that retards the migration of a portion of the probe (lane 6). This prolactin-inducible complex was further supershifted in the presence of specific anti-Stat5 antibody (lane 7), but not when purified, non-specific IgG was added (lane 8), indicating a specific induction of Stat5 binding in response to prolactin stimulation.

An identical experiment was performed (lanes 9-12) that used Ad-Δ713-*Stat5* instead of WT virus for comparison of biological function between the isoforms. Again,

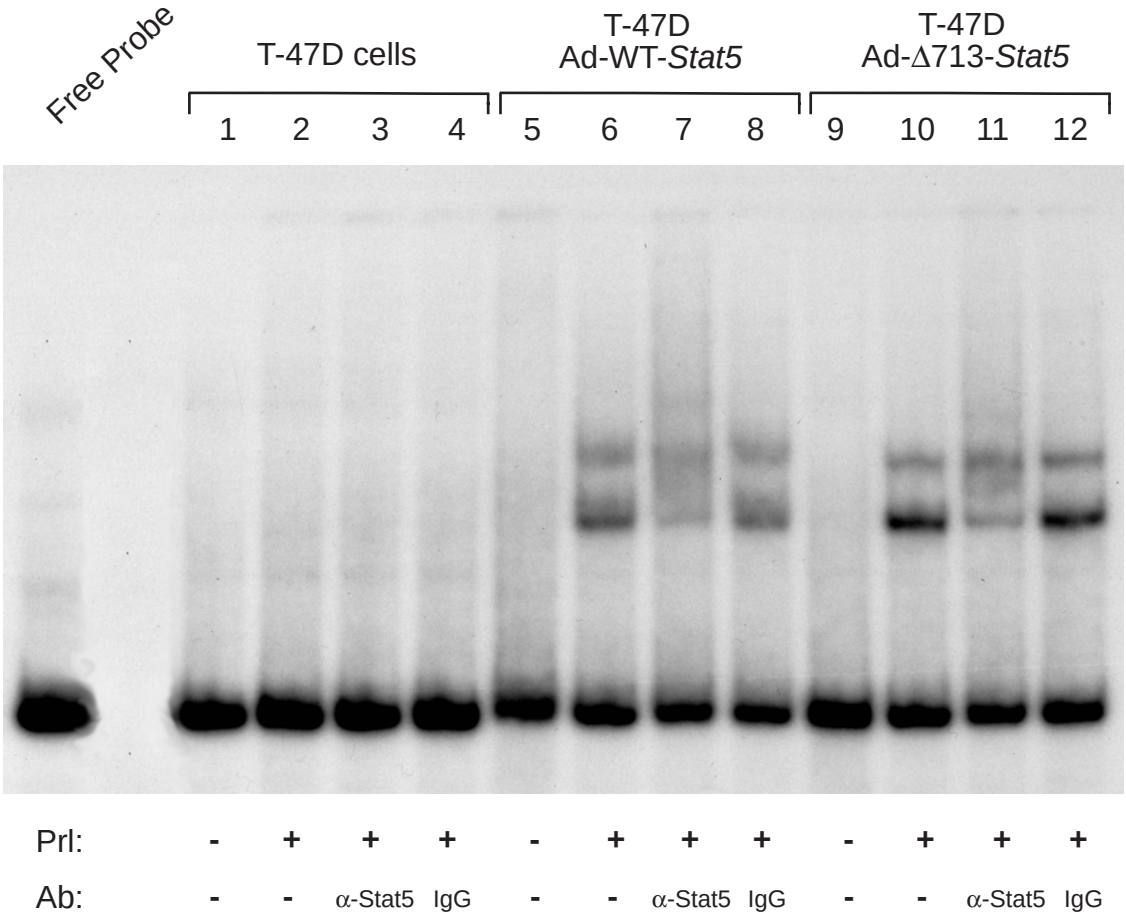


Figure 37. Prolactin activated Stat5 is able to associate with the cloned Stat5-response element fragment #29 as shown by EMSA and anti-Stat5 supershift. Cells were incubated with (+) or without (-) Prl for 30 min, then harvested as described to prepare nuclear extracts. Non-specific IgG was used as a negative supershift control. Stat5 activation and DNA binding in response to Prl stimulation is present in Ad-WT-*Stat5* and Ad-Δ713-*Stat5* infected T-47D human breast cancer cells (Lanes 5-12). Detectable Stat5 induction and binding was noted in the T-47D cells without adenoviral Stat5 added, but required increased nuclear extract (data not shown) while no activity was detected as shown (Lanes 1-4). Stat5-DNA complexes were supershifted in the presence of anti-Stat5 antibody (Lanes 7 and 11), but not in the presence of non-specific, purified IgG (Lanes 8 and 12). Note the increased binding affinity and higher mobility for the Δ713-Stat5 variant when compared to the WT isoform, consistent with previous reports.

specific and inducible Stat5 binding to the probe is definitively shown. Also note the slightly faster mobility of Δ713-Stat5 complex compared to WT. This is a result of the Δ713 variant of the Stat5 protein that has had 80 amino acids truncated from the C-terminal transactivation domain. Also, consistent with prior results in our lab, the Δ713-Stat5 isoform binds DNA tighter when compared to the WT protein (Yamashita *et al.* 2001), as exhibited in the strength of signal in lanes 10 and 12 compared to 6 and 8, respectively.

In conclusion, this figure illustrates the development of a technique to specifically test, in a high-throughput manner, a DNA fragment for the interaction with an inducible transcription factor. The specificity of Stat5 interaction with the fragment only after prolactin stimulation and supershift in the presence of Stat5 antibody verifies the effectiveness of this validation method.

Another fragment (fragment #80) was radiolabeled for a similar experiment as described for fragment #29 and shown in Figure 37. Figure 38 shows the nuclear extracts of Ad-WT-*Stat5* infected T-47D cells treated with or without prolactin and allowed to interact with the previously cloned probe. Specifically, prolactin induced a complex that bound and retarded the migration of the DNA fragment (lane 2) when compared to the nuclear extract of cells not stimulated with prolactin (lane 1). This complex was further supershifted by the addition of anti-Stat5 antibody (lane 3), but not with the addition of non-specific, purified IgG. Consistent with the previous experiment (fragment #29), T-47D cells that were not infected with an adenovirus that contained either WT- or Δ713-*Stat5* did not show a significant induction of Stat5 binding to the probe (data not shown). Furthermore, consistent with Figure 37 the nuclear extracts of cells infected with Ad-

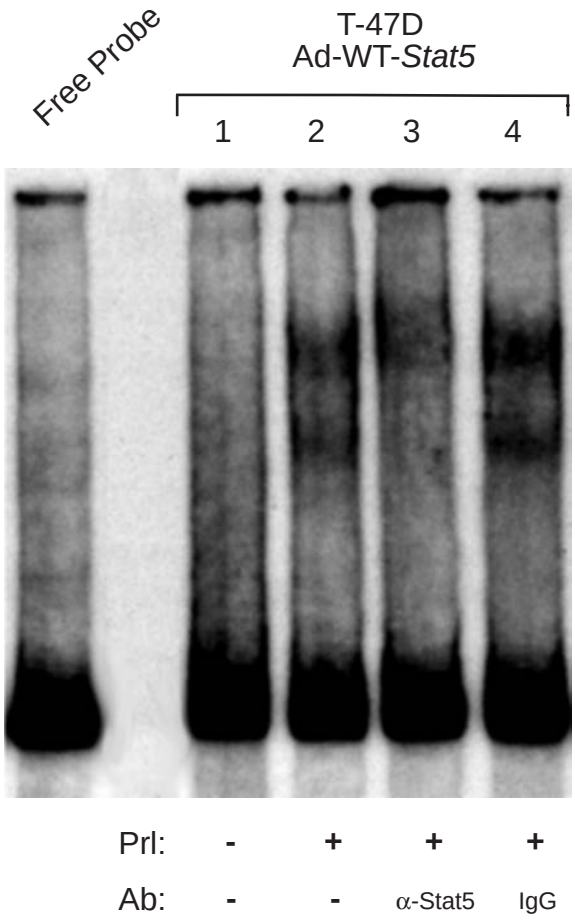


Figure 38. Prolactin activated Stat5 is able to associate with the cloned Stat5-response element fragment #80 as shown by EMSA and anti-Stat5 supershift. Cells were incubated with (+) or without (-) Prl for 30 min, then harvested as described to prepare nuclear extracts. Non-specific IgG was used as a negative supershift control. Stat5 activation and DNA binding in response to Prl stimulation is present in Ad-WT-*Stat5* (Lanes 1-4) and Ad-Δ713-*Stat5* (data not shown) infected T-47D human breast cancer cells. Detectable Stat5 induction and binding was noted in the T-47D cells without adenoviral Stat5 added, but required increased nuclear extract (data not shown). Stat5-DNA complexes were supershifted in the presence of anti-Stat5 antibody (Lane 3), but not in the presence of non-specific, purified IgG (Lane 4).

Δ713-Stat5 exhibited a stronger and more focused shift of radiolabeled probe when compared to WT (data not shown).

A third DNA fragment (fragment #138) was tested and showed a similar, specific induction of DNA binding to the cloned Stat5 response element after prolactin stimulation, as shown in Figure 39. Again, the results from T-47D cells where adenovirally delivered WT-*Stat5* was used are shown. Stat5 is also able to bind this fragment after prolactin stimulation, verifying the ability of Stat5 to interact with the cloned genomic element.

Protocol-Specific Adaptation of EMSA

Several of the steps required optimization from the basic EMSA protocol (Kirken *et al.* 1997), including: 1) testing various concentrations of polyacrylamide gels for optimal migration of complexes that are larger than normal, 2) establishing rapid isotope labeling protocol for PCR fragments, and 3) improving the sensitivity Stat5 binding of nuclear extracts of T-47D cells so that many fragments can be tested in parallel.

Given that most EMSA probes are synthesized to approximately 20 – 40 base pairs, a 5% non-denaturing poly-acrylamide gel can be used to allow the probe to migrate sufficiently through the matrix and allow differential migration due to protein interaction to be visualized. However, the length (~ 200 base pairs) of the cloned Stat5-chromatin interaction sites to be tested necessitated additional gels to be tested. The 3% gel utilized in Figures 37-39 provided the best differential migration between free probe, probe with activated Stat5 bound, and supershifted DNA-protein complexes. During the optimization of this segment of the protocol several different polyacrylamide gel

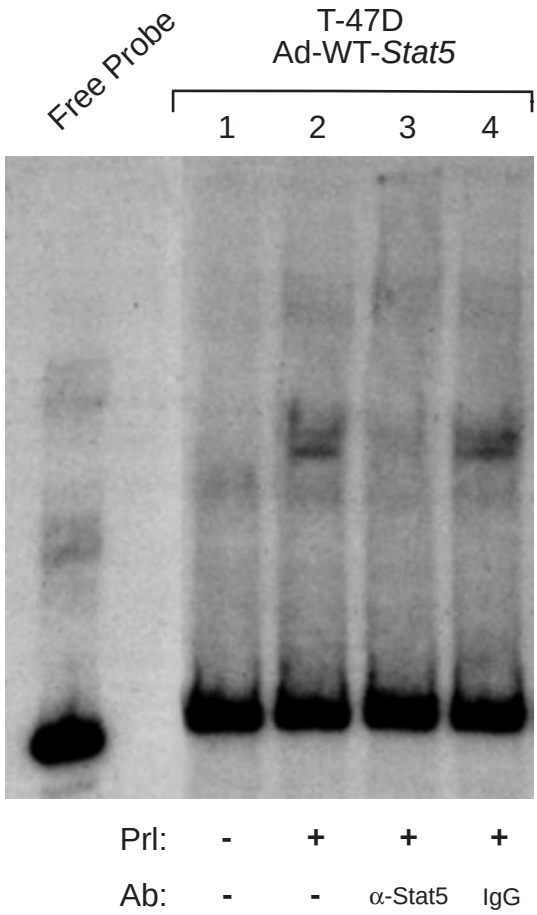


Figure 39. Prolactin activated Stat5 is able to associate with the cloned Stat5-response element fragment #138 as shown by EMSA and anti-Stat5 supershift. Cells were incubated with (+) or without (-) Prl for 30 min, then harvested as described to prepare nuclear extracts. Non-specific IgG was used as a negative supershift control. Stat5 activation and DNA binding in response to Prl stimulation is present in Ad-WT-*Stat5* (Lanes 1-4) and Ad-Δ713-*Stat5* (data not shown) infected T-47D human breast cancer cells. Detectable Stat5 induction and binding was noted in the T-47D cells without adenoviral Stat5 added, but required increased nuclear extract (data not shown). Stat5-DNA complexes were supershifted in the presence of anti-Stat5 antibody (Lane 3), but not in the presence of non-specific, purified IgG (Lane 4).

compositions were tested, however the 4%, 5%, and 6% gels inhibited the migration of the probe complexes into the matrix (data not shown).

Secondly, most other EMSA probe preparation requires the synthesis of complementary oligonucleotides that have been annealed, and end labeling of the 5' terminus using T4 Polynucleotide Kinase and [γ^{32}P] ATP (Kirken *et al.* 1997). For rapid screening of our fragments, the author instead used PCR to synthesize and label the probe. A small quantity of [α^{32}P] dATP was added with the other 4 dNTP nucleotides to the reaction mixture, the *Taq* Polymerase then randomly incorporated the radioactive dATP with unlabeled dATP for the strand synthesis in a standard PCR amplification reaction.

Verification of the specificity and synthesis of the PCR products was visualized by gel-electrophoresis, drying the gel on Whatmann paper, and then visualizing by autoradiography to determine the strength of signal and uniform product generation.

Lastly, to increase the sensitivity of nuclear T-47D extracts so that more samples could be screened in parallel in future high-throughput versions of this method, the author compared the Stat5-binding activities of nuclear extracts from prolactin-stimulated T-47D cells that had either been exposed to control conditions, adenovirus carrying WT-*Stat5*, or adenovirus carrying the hyperbinding Δ713-*Stat5* variant. As shown in Figure 37 the WT- and Δ713-Stat5 samples showed strong, specific, prolactin-induced Stat5 binding to the probe. While the control (no-infection) cells showed inducible binding with autoradiographic overexposure (data not shown), the amount of extract required and background levels reduced effective, high-throughput screening.

In conclusion, this validation procedure using EMSA to determine Stat5-binding capacity of the cloned fragments, showed that 3 out of 3 fragments tested indeed bound Stat5 specifically. This limited analysis proves the principle of the validation step and opens for further testing of additional cloned DNA fragments as they are isolated.

The results from this section conclusively establish the novel method described here as a specific and functional tool for cloning and identification of Stat5 binding sites within the human genome. The prolactin-inducibility of Stat5 binding when using known Stat5-responsive targets showed validation of the specificity of the immunoprecipitation and enrichment of Stat5 binding elements in the final recovered pool. Novel elements cloned by this method were shown to bind Stat5 in EMSA and were further supershifted by the addition of specific Stat5 antiserum.

Materials and Methods

As described in the *Introduction* section to this chapter, the materials and methods have been integrated throughout the *Results* section since the goal was the development and optimization of this novel methodology. The following is the current protocol used for the identification and validation of Stat5-chromatin interaction sites.

Optimized Chromatin Immunoprecipitation Protocol

1. Stimulate ~10^7 cells (confluent T175 flask) w/ 10 nM human Prl for 30 minutes
    - 1000X dilution (e.g. 25µl hPrl in 25 ml media)
2. Crosslink Stat5 to DNA by adding formaldehyde (Fisher Scientific, Fair Lawn, NJ) to medium at final concentration of 1% and incubate 30 minutes at 37°C

- Be sure to seal flask to eliminate contamination of incubator, use non-vented caps and seal tightly

3. Aspirate medium/formaldehyde, wash in 10 ml wash/scrape buffer (see below), aspirate, add 10 ml additional buffer and collect cells with cell scraper and collect in appropriately labeled 15 ml tube

    - Prepare wash/scrape buffer immediately previous to use, keep ice cold)
    - For 16 ea T175 flasks: (10 ml X (16x2)=320 + 10%= 352 ml):

	Stock Solution	**For 352 ml**
1 mM PMSF (Sigma-Aldrich, St. Louis, MO)	100 mM	3.5 ml
2 µg/ml Aprotinin (Sigma-Aldrich, St. Louis, MO)	2 mg/ml	352 µl
2 µg/ml Pepstatin A (Sigma-Aldrich, St. Louis, MO)	2 mg/ml	352 µl
Ice cold PBS		348.2 ml

4. Pellet cells for 4 minutes at 700 X *g* at 4°C and discard supernatant.

    - Cell pellets may be stored at –70°C
    - To continue, resuspend cell pellet in 400 µl SDS-lysis buffer (see below) and incubate on ice for 10 minutes
    - SDS-lysis buffer for 10 ml:

	Stock Solution	**For 10 ml**
1% SDS	10%	1 ml
10 mM EDTA	0.5 M	200 µl
50 mM Tris-HCL, pH 8.0	1 M	500 µl
dH2O		8.3 ml
		For 2 ml (Fresh)
1 mM PMSF	100 mM	20 µl
2 µg/ml Aprotinin	2 mg/ml	2 µl
2 µg/ml Pepstatin	2 mg/ml	2 µl

5. Transfer cell lysates to 1.5 ml microcentrifuge tube. Sonicate lysates to average DNA fragment length of approximately 400 bp. Cool lysates on wet ice for ~1' between pulses.
    - Fisher Scientific, Sonic Dismembranator Model 500 Sonicator: (Branson 450 sonicator) 2 X 30 seconds at 50% amplitude with the stepped microtip assembly. When sonicating make sure to put the end of the microtip as close to bottom of the tube (submerged) as possible. This will eliminate foaming and incomplete sonication. However, make sure the tip will not contact the inner wall of the tube.
    - Pellet debris by centrifugation for 10' at 13,200 RPM at 4°C
    - Transfer supernatant to a new, appropriately labeled 15 ml tube.
6. Dilute supernatant 10 fold in IP buffer (400 µL to 4 ml). After dilution keep 1% (40 µl) for future pre-IP analysis.
    - Add inhibitors immediately prior to use and keep ice cold:

      (3.6 X 4 = 14.4 ~ 15 ml)

	Stock Solution	*For 50 ml*
0.1% SDS	10%	500 µl
1.1% Triton X	100%	550µl
1.2 mM EDTA	0.5 M	120 µl
16.7 mM Tris-HCl, pH 8.1	1 M	835 µl
16.7 mM NaCl	5 M	167 µl
dH2O		47.8 ml
		For 15 ml (Fresh)
1 mM PMSF	100 mM	150 µl
2 µg/ml Aprotinin	2 mg/ml	15 µl
2 µg/ml Pepstatin	2 mg/ml	15 µl

7. Preclear chromatin solution with 80 µL 50% protein A-Sepharose treated beads for 30' at 4° C with end-over-end rotation

    - This step will improve overall specificity by removing proteins and DNA that will stick to the beads non-specifically

	Stock Solution	**For 1 ml**
Protein A-Sepharose beads (Amersham-Pharmacia, Piscataway, NJ)	600 µl	Pellet and remove supernatant
20 µg poly (dI.dC)-(dI.dC) (Amersham-Pharmacia, Piscataway, NJ)	1 mg/ml	20 µl
0.1% BSA	10 mg/ml	10 µl
TE, pH 7.4		600 µl (to 1 ml)

8. Pellet beads by centrifugation and collect supernatant (4000 RPM for 5')

    - Transfer supernatant to new 1.5 ml microcentrifuge tubes, 1 ml / tube

9. Add appropriate 1° antibody to supernatant and incubate overnight at 4° C with rotation.

    - 5 µl (200 ng/µl) N-terminal antibody to proper tube
    - 1 µl (1000 ng/µl) IgG 2a, kappa pre-immune IP control

10. Collect immune complexes with preincubated beads (as previous) add 30 µl per microcentrifuge tube and incubate for 1 hour at 4° C with rotation
11. Pellet beads by centrifugation (5000 RPM for 20 seconds at 4°C)
    - Discard supernatant (immune complexes can be frozen at -70°C if necessary)
    - Wash beads in each tube with each buffer (A, B, C), 1 ml per wash
    - Using first wash – combine similar tubes, (e.g. split 1 ml into 2 and add 0.5 ml into each identical treatment/IP tube then combine into 1 tube to half total number of tubes)
    - Add 1 ml buffer, incubate 5' (invert tube occasionally)
    - Spin 2' at 1000 RPM, at 4°C
    - Discard supernatant
    - Wash buffer D/E (TE, pH 8.0), once with each buffer

Wash Buffer A

	Stock Solution	For 45 ml
0.1% SDS	10%	450 µl
1% Triton X	100%	450 µl
2 mM EDTA	0.5 M	180 µl
20 mM Tris-HCl, pH 8.0	1 M	900 µl
150 mM NaCl	5 M	1.350 ml
dH2O		41.6 ml

Wash Buffer B

	Stock Solution	For 45 ml
0.1% SDS	10%	450 µl
1% Triton X	100%	450 µl
2 mM EDTA	0.5 M	180 µl
20 mM Tris-HCl, pH 8.0	1 M	900 µl
500 mM NaCl	5 M	4.5 ml
dH2O		38.5 ml

Wash Buffer C

	Stock Solution	For 45 ml
0.25 M LiCl	5 M	2.25 ml
1% NP-40	10%	4.5 ml
1% Na-deoxycholate	10%	4.5 ml
10 mM Tris-HCl, pH 8.0	1 M	450 µl
1 mM EDTA	0.5 M	90 µl
dH2O		33.2 ml

12. Elute immune complexes from protein A Sepharose beads by adding 250 µl fresh elution buffer (see below), vortex gently and incubate at room temperature for 15' with rotation. Spin down beads and transfer supernatant to fresh tube. Repeat and combine elutes.

	Stock Solution	*For 10 ml*
1% SDS	10%	1 ml
0.1 M $NaHCO_3$	1 M	1 ml
dH2O		8 ml

13. Add 20 µl of 5 M NaCl to each tube of the recovered elutate to reverse crosslinks. Incubate samples overnight at 65°C (gives 0.2 M NaCl final dilution)

14. Add protease solution for digestion of proteins (DNA bound Stat5), incubate 1 hour at 45°C with shaking

    - 10 µl 0.5 M EDTA
    - 20 µl 1 M Tris-HCl, pH 7.6
    - 2 µl 10 mg/ml Proteinase K (Sigma-Aldrich, St. Louis, MO)

15. Phenol:Chloroform extraction to recover DNA fragments

    - Add 1 volume (450 µl) Phenol:Chloroform:Isoamylalcohol (25:24:1 v/v) (Invitrogen, Carlsbad, CA), vortex 10 seconds, spin 2 minutes at 13,200 RPM, transfer top (aqueous) phase to fresh tube.

- Add 1 volume (450 µl) Phenol:Chloroform:Isoamylalcohol, vortex 10 seconds, spin 2 minutes at 13,200 RPM, transfer top (aqueous) phase to fresh tube.
- Add 1 volume (450 µl) Chloroform (Fisher Scientific, Fair Lawn, NJ) vortex 10 seconds, spin 2 minutes at 13,200 RPM, transfer top (aqueous) phase to fresh tube.
- Add 1/10 volume (45 µl) of 3 M sodium acetate (Quality Biological, Gaithersburg, MD), vortex
- Add 1 µl glycogen (Invitrogen, Carlsbad, CA) (20 µg/µl), vortex
- Add 2 volumes (900 µl) 100% ethanol, vortex
- Precipitate overnight at -20°C
- Spin samples at 13,200 RPM for 15 minutes at 4°C
- Remove supernatant by suction taking care not to disturb the pellet
- Wash samples in 2 volumes 80% ethanol (900 µl)
- Spin samples at 13,200 RPM for 15 minutes at 4°C
- Remove supernatant by suction taking care not to disturb the pellet
- Air dry tubes 15 minutes +
- Resuspend DNA pellet in 30 µl TE, pH 8.0

16. Samples may then be used for PCR analysis for Stat5 association with known response elements or may be manipulated further for cloning and identification.
17. T4 DNA polymerase (New England Biolabs, Beverly, MA) is used with standard conditions to generate blunt ends on all DNA fragments, since sonication produces a random assortment of blunt and protruding ends.

- 5 µl of immunoprecipitated product
- 2 µl 10X reaction buffer [33 mM Tris-acetate, 66 mM K-acetate, 10 mM Mg-acetate, and 0.1 mg/ml BSA]
- 10 µl 1.0 mM DTT
- 1.6 µl 10 mM dNTP
- 1.4 µl dH_2O
- The samples were incubated at 37°C for 5 minutes then diluted to 200 µl with water.

18. The blunted fragments are then recovered by phenol:chloroform extraction (as described above), followed by ethanol precipitation and resuspended in 30 µl dH_2O.
19. *Taq* DNA polymerase (New England Biolabs, Beverly, MA) is then used to add a 3' Adenosine nucleotide to each double stranded DNA fragment. A standard 25 µl reaction is used and is incubated at 72°C for 15 minutes.
    - 10 µl of blunted, immunoprecipitated sample
    - 2.5 µl of 10X PCR reaction buffer
    - 2.5 µl 25 mM $MgCl_2$
    - 4 µl 10 mM dNTP
    - 0.3 µl *Taq* polymerase
    - 5.7 µl H_2O
20. The DNA fragments with the 3' Adenosine overhang are then cloned into a bacterial expression vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA)

following the manufacturer's protocol, mixed and incubated 30 minutes at room temperature, then transferred to ice.

- 4.0 µl previous product
- 1.0 µl dilute salt solution (from kit)
- 1.0 µl TOPO vector

21. The ligated vector is then transformed into highly efficient electrocompetent bacteria, DH5αe (Invitrogen, Carlsbad, CA), under standard, recommended conditions.
    - 1.5 µl of TOPO TA ligation (previous step)
    - 25 µl DH5αe electrocompetent bacteria
22. After transformation the cells are plated on S-Gal (Sigma-Aldrich, St. Louis, MO) enriched agar plates and grown overnight at 37°C. Individual colonies are purified, analyzed, and documented as described.

EMSA Validation of Prospective Novel Stat5 Binding Sites

Generation of adenovirus for gene delivery of dominant-negative and wild-type Stat5:

Expression vector for murine Stat5a (pXM-Stat5a) was kindly provided by Xiuwen Liu and Lothar Hennighausen (National Institutes of Health, Bethesda, MD) (Liu *et al.* 1995). A dominant-negative (DN) variant of Stat5 (Stat5aΔ713) was derived by truncation after amino acid residue Ala713 of pXM-Stat5a, using a PCR fragment generated using 5' TAA TAC GAC TCA CTA TAG GG 3' (sense) and 5' GCT CTA GAC TAG GCA TCT GTG GAT GCA TTG 3' (antisense) primers, followed by *EcoR*I and *Xba*I digestion, and subcloning into the *EcoR*I-*Xba*I-digested pXM-Stat5a. The DNA sequence of the resulting construct pXM-Stat5aΔ713 was verified before use. The

ability of our DN-Stat5 (Stat5aΔ713) expression construct to completely suppress both Stat5a- and Stat5b-mediated transcriptional activation has been reported (Yamashita *et al.* 2003). Replication-defective human adenovirus (Ad5) carrying wild-type Stat5 (WT-Stat5) or DN-Stat5 was generated using the AdEasy Vector system (QBIOgene, Carlsbad, CA). The open reading frame sequences of DN-Stat5 and WT-Stat5 were released from respective plasmids by 1) digestion with *EcoR*I, 2) blunt-ending with Klenow DNA polymerase, and 3) digestion with *Hind*III, and the resulting fragments were subcloned into the Klenow DNA polymerase blunt-ended *Bgl*II site and the unmodified *Hind*III site of the pShuttle-CMV transfer vector. Homologous recombination of WT-Stat5 or DN-Stat5 transfer vectors with the pAdEasy vector was performed in BJ5183 *E. coli* by electroporation. Recombined clones were screened by Kanamycin-resistant growth, and confirmed by *Pac*I digestion to yeield two bands of 30 kb and 4.5 kb. The recombinant viruses were packaged in QBI-293A cells and resulting clones were selected from plaques and amplified. Expression of WT-Stat5 and DN-Stat5 from adenoviral stocks was verified by Western blotting using an anti-panStat5 antibody (Transduction Laboratories, Lexington, KY). Selected recombinant viral stocks were expaned in large-scale cultures, purified by double cesium chloride gradient centrifugation, and titered side-by-side by a standard plaque assay method in QBI-293A cells, as per the manufacturer's recommendations (Ahonen *et al.* 2003).

Preparation of cellular extracts for EMSA:

After reaching confluence in a T-75 cm^2 culture flask in growth medium, T-47D cells were infected with adenovirus containing Ad-WT-Stat5 or Ad-DN-Stat5 at an MOI = 6.67 according to standard procedures as described earlier. Parallel samples of T-47D

cells were not exposed to adenovirus as a standard control. After infection the cells were cultured in serum-free medium for 24 hours prior to hormone treatment. Cells were then stimulated for 30 minutes with human prolactin (10 nM). The culture medium was removed, and the cells were dislodged from the culture flask by scraping in ice-cold PBS with inhibitors, as described above. The cells were pelleted by centrifugation and the supernatant was removed and the cell pellets were frozen and stored at -80°C. When needed the pellets were immediately solubilized in 100 µl EMSA lysis buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 1 mM $MgCl_2$, 20% glycerol, 0.1% NP-40, 1 mM orthovanadate, 25mM NaF, 200 mM PMSF, 0.5 mM DTT, 5 mg/ml aprotinin, 1 mg/ml pepstatin A, and 2 mg/ml leupeptin). Lysates were incubated on ice for 10 minutes and then clarified by centrifugation at 5,000 RPM for 2 minutes at 4°C. The supernatant was transferred to a new tube, centrifuged at maximum speed for 10 minutes at 4°C and labeled as cytoplasmic extracts and frozen. The pelleted nuclei (from the original tubes) were then vortexed briefly and then resuspended in 33 µl nuclei lysis buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 1 mM $MgCl_2$, 20% glycerol, 0.1% NP-40, 300 mM NaCl, 1 mM orthovanadate, 25mM NaF, 200 mM PMSF, 0.5 mM DTT, 5 mg/ml aprotinin, 1 mg/ml pepstatin A and 2 mg/ml leupeptin), vortexed again, and were stored on ice for 30 minutes then centrifuged for 20 minutes at maximum speed at 4°C. The supernatants were transferred to a fresh tube and were diluted with an equal volume of EMSA lysis buffer and stored as nuclear extracts at -80°C.

Generation of radiolabeled DNA probes:

The radiolabeled products were generated by PCR using the following parameters. For a 10 µl reaction: 5.0 µl Qiagen PCR master mix, 6.0 pmol M13 reverse primer, 6.0 pmol T7 primer, 1.0 µl appropriate PCR template (diluted 1:1000), and 0.25 µl α^{32}P dATP (10 mCi/ml). Initially the samples were incubated at 94°C for 1 minute, then cycled 36 times at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 20 seconds. The reaction was then held at 72°C for 5 minutes following the cycling to allow for product fill-in and addition of a 3' terminal "A". After completion of the cycling, the PCR products were purified using the Qiagen PCR purification kit, according to manufacturer's instructions. The final products were eluted in 50 µl and stored at -20°C until use.

DNA-protein binding reaction:

For the electrophoretic mobility shift assay (Wilson, et al., 1992), 1 ng of double-stranded oligonucleotide probe corresponding to the cloned genomic response element was labeled by incorporating radioactive adenosine nucleotide into the PCR product, as described above. The DNA-protein binding reactions were performed in a 10 µl mixture containing 5 µl of nuclear extract from the respective sample, 1 µg of double-stranded poly dI:dC (Boehringer Mannheim, Indianapolis, IN) in 10 mM Tris-HCl (pH 7.4), 100 mM KCl, 1 mM 1,4-dithiothreitol (DTT), 10% glycerol, and 5 mM $MgCl_2$. A 5X binding buffer concentrate (50 mM Tris-HCl (pH 7.4), 500 mM KCl, 5 mM 1,4-dithiothreitol (DTT), 50% glycerol, and 25 mM $MgCl_2$) was prepared at stored at -20°C then diluted to the above concentrations. After 1 h on ice, samples (with 1 ng

specific anti-Stat5 antibody (Upstate Biotechnology), or 1 ng non-specific, purified IgG (Sigma), or no antibody) were incubated with 2.0 µl ^{32}P-labeled PCR probe and incubated for 20 min at room temperature. The samples were then loaded with a 0.5X dilution of DNA loading buffer with bromophenol blue. The samples were then resolved by non-denaturing polyacrylamide gel electrophoresis.

EMSA analysis of protein binding to PCR radiolabeled probe:

A 3% native resolving gel mixture was made from 0.6 ml 10X TBE buffer (890 mM Tris-borate, pH 8.3, 890 mM boric acid, 20 mM EDTA; Quality Biological, Gaithersburg, MD;), 2.5 ml of 30% acrylamide stock, 2.5 ml of 50% glycerol, 20.5 ml deionized water, 125 ml of 10% ammonium persulfate (APS), and 25 ml of TEMED. The gel was mixed and poured carefully and bubbles were eliminated by gently tapping the glass plates allowing them to rise to the top. A teflon comb was inserted in the top of the gel with care being taken not to create air bubbles, and the gel polymerized for 1 h. The gel was gently mounted in the electrophoresis apparatus and bubbles removed from between the bottom plates. The upper and lower buffer chambers were filled with 0.25X TBE electrophoresis buffer, the comb was removed, and the gel pre-run in 0.25x TBE buffer at 4-10°C for 1.5 hour at 300 V. Following this equilibration, 10 ml of sample was loaded per well; blank wells were filled with 60 ml of EMSA blank buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1,5 mM MgCl2, 1 mM sodium vanadate, 25 mM sodium fluoride, 10 mM sodium pyrophosphate in 0.5X loading buffer). After loading of samples, the gels were run at room temperature for approximately 2 hours at 200 V. Gels were dried on 3mm Whatmann paper by heating under vacuum using a slab dryer

(Thermo-Savant, SG210D gel dryer) and exposed to X-Omat XAR-5 autoradiography film with an intensifying screen at various time points, from 2 hours to overnight at -70°C.

Supershift analysis:

For supershift analysis, before addition of ^{32}P-labeled probe, extracts were incubated with antibody for 30 min on ice as described previously.

CHAPTER III:

STRATEGY TO IDENTIFY STAT5 INDUCED TRANSCRIPTS BASED ON DOMINANT-NEGATIVE DIFFERENTIAL SUPPRESSION OF TRANSCRIPTION AND RNA GENE CHIP ANALYSIS

Introduction

As detailed and cited thoroughly in the *Background* section, the analysis and characterization of individual mRNA transcripts in cells and tissues has greatly improved our understanding of the organization, structure, and function of the human genome. While several different molecular biology techniques have been utilized in the compilation of mRNA-related discovery of genes and gene function, the emergence of large-scale gene chip analysis now provides unparalleled data generation. Gene chip studies provide a reproducible framework with high sensitivity, specificity, and high-throughput when compared to other mRNA analyses.

There are, nonetheless, several limitations associated with gene chip or microarray technology as detailed and cited in the *Background* section. First, in contrast to the chromatin-interaction based approach presented above, large-scale RNA array analysis is biased toward the detection of highly abundant gene transcripts that increase or decrease markedly. Second, as of yet there are no absolute collections of genes synthesized on one chip or a series of chips, although genome-wide microarrays for RNA analysis will soon be a reality. Third, gene expression comparison between two experimental conditions is frequently hampered by the inability to differentiate between genes regulated directly or indirectly by a given transcription factor. Specifically, the initial induction of a transcript by the transcription factor of interest rapidly leads to

induction of secondary genes. For instance, it is virtually impossible to identify Stat5-regulated genes by comparing mammary tissue from Stat5-null mice to tissue from wild type mice, because the transcript pools reflect so many secondary events. In order to effectively take advantage of large-scale gene expression analysis to identify genes directly regulated by Stat5, the experimental strategy needs to be carefully considered. Below the author presents new methodology that will allow rapid identification of Stat5 target genes.

The author has used an innovative approach to specifically distinguish Stat5-regulated transcripts in human T-47D breast cancer cells. The author and Dr. Rui have termed the technology dominant-negative differential suppression of transcription, which when combined with large-scale RNA gene chip analysis allows powerful study and identification of Stat5 regulated transcripts. Briefly, the strategy employs a dominant-negative mutant of Stat5 to differentially suppress prolactin-induced Stat5-dependent gene expression, while not affecting prolactin-induced, Stat5-independent genes. Sensitivity of prolactin-induced genes to dominant-negative suppression will distinguish Stat5 target genes from Stat5-independent genes.

A key to success of this strategy is to keep the Stat5-activation time short, with a strict focus on immediate-early gene responses, before secondary gene changes begin to predominate. The author has therefore restricted the experimental induction time to the first 5 h of Stat5 activation.

In order to effectively introduce a dominant-negative Stat5 molecule into the entire population of cultured breast cancer cells, the author used adenoviral gene delivery. Several functional studies of this naturally occurring variant, as well as the adenoviral

delivery method are described elsewhere (Ahonen *et al.* 2003; Xie *et al.* 2002; Yamashita *et al.* 2001) and in this dissertation (Figure 37). To further enhance wild-type Stat5-mediated signal and gene induction in control cultures, the author introduced wild type Stat5 by adenoviral delivery. Furthermore, because glucocorticoid hormones cooperate with Stat5 to induce breast epithelial cell differentiation (Doppler, Groner, and Ball 1989; Groner, Altiok, and Meier 1994; Juergens *et al.* 1965), the author also included a pretreatment period of confluent cultures of T-47D cells with dexamethasone (Dex, 1 uM, 70 h) (Schaber 1998) Unpublished observations of Dr. Hallgeir Rui's laboratory. Adenoviral delivery of wild type or dominant-negative Stat5 was initiated 40 h prior to initiation of Stat5 activation with prolactin (10 nM, 5 h).

After treatment with or without prolactin for 5 h, T-47D cells that had been preinfected with adenovirus carrying either wild type or dominant-negative Stat5, were harvested and RNA was isolated as described in the *Materials and Methods* section. Three independent experiments were carried out to permit statistical evaluation of the data and the mRNA samples were analyzed using the Affymetrix U133A gene chip as described in the *Materials and Methods* section. The U133A chip allows simultaneous analysis of approximately 22,000 human transcripts.

As will be demonstrated below, the strategy successfully distinguished between prolactin-induced, Stat5-dependent genes from prolactin-induced, Stat5-independent genes. Specifically, genes upregulated within 5 h of prolactin stimulation in Ad-WT-Stat5 treated cells, but not in Ad-DN-Stat5 treated cells, represent Stat5 regulated genes. In contrast, genes that were upregulated by prolactin in both Ad-WT-Stat5 and Ad-DN-Stat5 represent Stat5-independent, prolactin-induced genes. Such Stat5-independent

genes may be induced by other Stat transcription factors, e.g. Stat1 or Stat3, which prolactin may also activate in T-47D cells (Schaber *et al.* 1998).

Results/Discussion

Prolactin-Induced, Stat5-Mediated Gene Transcription

Confluent T-47D human breast cancer cells can be induced to phenotypically differentiate in response to glucocorticoid treatment (Ball *et al.* 1988; Doppler, Groner, and Ball 1989). Since Stat5 has been shown to be critically important in mammary growth and differentiation (Liu *et al.* 1997; Teglund *et al.* 1998), the present work used this model to specifically identify Stat5 regulated genes. As previously described the author used adenoviral delivery of WT- or DN-Stat5 to specifically characterize genes with respect to prolactin-induced Stat5 activation. In particular, the ability of DN-Stat5 to bind Stat5 response elements, but inhibit transcription, provides a powerful tool for identification of Stat5-specific transcripts when compared to WT-induced transcripts.

The cells were prepared as described in the *Materials and Methods* section and the RNA was harvested and verified for quality before cRNA labeled probes were generated. All expression data was generated using Affymetrix 5.0 ArraySuite software. After the raw data was generated a two-group comparison was performed to estimate the fold change between the (-) prolactin samples and the (+) prolactin samples and statistically determine the significance between the triplicate samples, as described in the *Materials and Methods* section. Therefore a value of 1.0 represents no change in gene expression between (-) prolactin samples and (+) prolactin samples.

Table 5 specifies examples of genes that were identified as Stat5 target genes by this strategy, since their relative expression levels increased when stimulated with prolactin in the Ad-WT-Stat5 treated cells, but remained unchanged or decreased in theAd-DN-Stat5 cells. The examples of Stat5 target genes include a transcription factor (*ETS variant gene 6*, frequently altered in human leukemias), a transmembrane receptor of the tetraspannin superfamily (*member 7* – forms complexes with integrins and other cell surface proteins), the regulatory subunit of protein kinase A (β subunit, metabolic stress-sensing kinase), *Calpain* (a protease involved in proteasomal degradation and apoptosis), *Harakiri* (a BCL2 interacting protein involved in apoptosis regulation), a lysyl oxidase gene (*lysyl oxidase-like 2*, initiates extracellular crosslinking of collagens and elastin), and *interferon α-16*. While it is beyond the scope of this work to discuss the potential role of these factors in Stat5-induced differentiation of breast cancer cells, these gene examples provide proof-of-principle of the described strategy to identify Stat5-regulated transcripts. In contrast, several genes were induced by prolactin but were not dependent on Stat5. An example of such a gene is *Selenoprotein X* (conserved Selenium binding protein), which was induced more than 3-fold in by prolactin in both the presence and absence of dominant-negative Stat5 (Table 5).

Table 5. Summary of Stat5-Regulated Genes by Dominant-Negative Differential Suppression of Transcription by RNA Gene Chip Analysis

	Prl-induction WT-Stat5 Fold Change	*Prl-Induction DN-Stat5 Fold Change*
fibrinogen, A alpha polypeptide	**3.5 (p=0.01)**	**-2.1 (p>0.05)**
DKFZP434B168 protein	**3.4 (p=0.05)**	**-1.2 (p>0.05)**
harakiri, BCL2 interacting protein (contains only BH3 domain)	**3.4 (p=0.02)**	**1.2 (p>0.05)**
lysyl oxidase-like 2	**3.4 (p=0.01)**	**1.2 (p>0.05)**
calpain 5	**3.2 (p=0.03)**	**1.0 (p>0.05)**
ets variant gene 6 (TEL oncogene)	**3.2 (p=0.03)**	**-1.3 (p>0.05)**
hypothetical protein PRO2198	**2.5 (p=0.001)**	**1.0 (p>0.05)**
transmembrane 4 superfamily member tetraspan NET-7	**2.3 (p=0.01)**	**-1.7 (p>0.05)**
protein kinase, AMP-activated, beta 1 non-catalytic subunit	**2.0 (p=0.005)**	**-1.3 (p>0.05)**
interferon, alpha 16	**2.1 (p=0.02)**	**1.3 (p>0.05)**
selenoprotein X, 1	**3.2 (p=0.001)**	**4.0 (p=0.002)**

Further work is needed to identify the Stat5 response elements in the promoters of Stat5-dependent genes and to determine whether they are important for Stat5-induced differentiation growth inhibition and metastatic invasion of breast cancer cells.

Materials and Methods

Cell Culture and RNA Extraction

Cultures of T-47D human breast cancer cells were grown to confluence in T-75 cm^2 flasks as described previously, the medium was then changed to DMEM supplemented with 10% FBS and 1 μM dexamethasone (Dex). After 24 hours the cells were then infected with Ad-WT-Stat5 or Ad-DN-Stat5 at an MOI = 6.67 in serum free medium for 90 minutes under standard conditions. Next the cells were incubated for 16 hours at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS and 1 μM Dex. The cells were then starved with serum-free DMEM and 1 μM Dex for 24 hours. After the 40 hour post-infection time period described above the cells were treated with or without 10 nM hPrl for 5 hours. The 5 hour stimulation time period was established to maximize direct Stat5 target genes and minimize the effect of secondary or indirect effects from the Prl-stimulation. All experiments were performed in triplicate for each experimental group.

For RNA harvest all procedures were performed according to the instructions from Affymetrix (Santa Clara, CA). First, after verifying good cell viability, the RNA was collected from each respective culture flask by using Trizol (Invitrogen) according to the manufacturer's recommended protocol for a cell culture flask area of 75 cm^2. The final RNA pellet was resuspended in 100 μl of RNase-free water. In order to get the

highest quality RNA for analysis the author performed a second, cleanup reaction to further purify the total RNA harvest. A Qiagen RNeasy Mini Kit (Valencia, CA) was used for column purification of the RNA and was utilized according to the manufacturer's recommended protocol. The final elution was performed twice at 30 µl each time for a total volume of 60 µl. The quality of RNA was analyzed with a UV Spectrophotometer (BioRad Labortories, Hercules, CA) after dilution in 10 mM Tris-HCl, pH 7.6 which gives a more accurate representation of RNA concentration and quality versus water. The A_{260}/A_{280} absorbance ratio for all samples was approximately 2.1, indicating a pure RNA sample. The samples were then diluted in RNase-free water to a final concentration of 1 µg/µl for future analysis. Additionally an Agilent BioAnalyzer 2100 (Palo Alto, CA) was used to determine the status of the RNA by detecting shifts in size due to degradation of RNA. After verification of good quality RNA the next steps were taken.

Preparation of Labeled cRNA and Array Hybridization

RNA was converted into a double-stranded cDNA by using an oligo-dT primer with a T7 promoter at the 5' end and the SuperScript Choice system for cDNA synthesis (Life Technologies, Inc.). Double-stranded cDNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in RNase-free water. A portion of the cDNA was used for *in vitro* transcription with a T7 RNA polymerase Megascript system (Ambion, Inc.) in the presence of biotinylated UTP and CTP (Enzo, Farmingdale, NY). The labeled cRNA was purified with Qiagen RNeasy columns, fragmented in the presence of heat and Mg^{++} as part of the hybridization cocktail. The

probe was quality checked again with an Agilent BioAnalyzer 2100 then hybridized to a Affymetrix Test3 chip to verify the quality of the probe and ability to hybridize before hybridizing to a specific human gene chip. The samples passed all screening tests and were hybridized to the Affymetrix human GeneChip U133A which contains 13,049 gene sequences characterized in terms of function and disease association and probes 14,593 UniGene clusters. After washing and staining, the arrays were scanned using a laser scanner controlled by the Affymetrix 5.0 ArraySuite software. The software employs statistical algorithms to calculate the quantitative value (signal intensity) and a qualitative value (present or absent) for each transcript on the array. Probe preparation, hybridization, scanning, and data compilation was done in accordance with the Georgetown University, Lombardi Cancer Center Microarray Core Facility (Washington DC).

Two-Group Comparison Statistical Analysis

The 2-group comparison is performed on values recorded in the Affymetrix 5.0 ArraySuite software and the data are transformed as logarithmic base 2 values. Using the log-transformed scale a mean is calculated for each gene within each group and a 2-sample, 2-sided t-test is conducted to test the mean values. The p-value indicates the significance of this test. The log-transformed mean for each group is then inverse-transformed to provide a geometrical mean as an overall estimate of expression in each group. Fold change is then calculated as the ratio of overall expression values from the 2 groups. The higher overall expression is divided by the lower expression. If the control group value is lower than the experimental group the fold change is given a negative value.

CHAPTER IV:

CELL DIFFERENTIATION-DEPENDENT CHANGES IN ACCESSIBILITY OF SPECIFIC GENOMIC RESPONSE ELEMENTS TO TRANSCRIPTION FACTOR STAT5

Introduction

Rationale for experiment

Virtually all normal, diploid human cells contain the same genome, yet different cell types vary phenotypically due in large part to highly regulated changes in chromatin structure. Likewise, different differentiation stages of the same cell type are thought to result from chromatin-specific changes that regulate gene regulatory programs through selective gene silencing. Thus, transcription factor access to specific regulatory interaction sites will differ between different stages of cell differentiation.

Cancer cells typically are aneuploid with variable losses or gains of chromatin, and, therefore, differ in this respect from normal cells due to loss of genomic stability (Lengauer, Kinzler, and Vogelstein 1998). Nonetheless, many cancer cells maintain the ability to undergo some extent of differentiation, and the general rules of chromatin regulation are also expected to apply to cancer cells.

Experiments performed in our lab and others have shown the ability of the human breast cancer cell line T-47D to phenotypically differentiate following the addition of glucocorticoids (Groner, Altiok, and Meier 1994; Lippman, Bolan, and Huff 1976; Schaber 1998). This change in cellular function is presumably a result of an alteration of gene expression (Doppler *et al.* 1990; Groner, Altiok, and Meier 1994), with associated

changes in accessibility of specific regions within the genome to be available for transcriptional regulation.

The author wanted to test the hypothesis that glucocorticoid-induced differentiation of breast cancer cells involves qualitative changes in patterns of access of transcription factors to known Stat5 interaction sites. Specifically, the authror hypothesized that the pattern of accessible chromatin-interaction sites for Stat5 change so that certain sites become accessible, other sites become inaccessible, and that some previously fully accessible sites would be less affected by glucocorticoid regulation. Alternatively, one could envision a simple accumulation of additional Stat5 interaction sites as a result of glucocorticoid-induced accessibility.

To test this hypothesis, cultures of T-47D cells were treated with or without 1 μM dexamethasone (Dex), a synthetic analogue of cortisol (the most potent glucocorticoid produced in the adrenal cortex) for 96 hours. After Dex pretreatment, cells were stimulated with or without human prolactin to activate Stat5. The cells were fixed by formaldehyde fixation and the method for capture of Stat5-bound genomic response elements was carried out as previously described. The final enriched pools of Stat5-bound DNA fragments were then amplified by PCR, using primer pairs designed to flank known Stat5 response elements within the promoter regions of a panel of known Stat5-regulated genes. A total of 12 known Stat5 response elements were tested: *αS1-Casein, α2-Macroglobulin, β-Casein, BCL-X_L, CIS-1, Cyclin D1, Estrogen Receptor α, IL-2Rα, OSM, CDKN1A (or $P21^{WAF1/CIP1}$), PIM1, and PRLR*. Background on each gene and the result of our Stat5 interaction analysis (Chromatin immunoprecipitation or "ChIP") are described below. Furthermore, each experiment contained a positive and negative PCR

control, as described in the *Materials and Methods* section, as well as negative immunoprecipitation controls. The amplifications for negative-immunoprecipitation samples were all negative for significant products, but were excluded from the figures for ease of viewing, except in the example provided in Figure 44 for *CIS1*.

Results

Casein protein family

The caseins represent a diverging group of multiple proteins in mammals (Dayhoff 1976). The differences in casein proteins were initially identified by differential migration using urea starch electrophoresis and were termed α (alpha), which is further subdivided into S1 and S2, β (beta), and κ (kappa) caseins (Jenness 1985). Further analyses showed that all 4 casein family members are co-localized to a locus on human chromosome 4q21.1 spanning approximately 350 kb (Chen, Bejcek, and Kersey 1995; Fujiwara *et al.* 1997; Rijnkels *et al.* 1997). It should be noted that the function of caseins in human milk is not restricted to providing a source of essential amino acids, as it has been shown that the caseins are also involved in the transport of calcium and phosphorous to the infant (Lonnerdal 1985; Lonnerdal, Bell, and Keen 1985).

αS1-Casein

αS1-Casein is one of many protein constituents of milk and is a variable component in expression between mammalian species (Womack and Threadgill 1990). In humans, it contributes to a relatively low proportion of the total protein, but this protein is very highly expressed in bovine milk (Lonnerdal and Forsum 1985; Womack

and Threadgill 1990). Since the promoter for human *αS1-Casein* contains a consensus Stat5 binding site, the author investigated whether Stat5 could associate with this promoter in T-47D in a glucocorticoid-dependent manner.

As seen in Figure 40, Dex treatment alters the accessibility of the *αS1-Casein* promoter for Stat5. Specifically, only after 96 hours of Dex treatment could Stat5 interact with this promoter in T-47D cells. In the samples starved for 96 hours in serum-free medium Stat5 was not able to bind to the promoter, even with prolactin stimulation. This change in binding patterns was presumably due to the alteration of chromatin conformation in response to the pro-differentiation effects of Dex on the T-47D cells.

It is interesting to note that also without prolactin stimulation, Stat5 could associate to some extent with the promoter for *αS1-Casein* when pretreated with Dex. One possible explanation is a cooperative binding of glucocorticoid receptor (GR), which is activated by Dex and Stat5 at the promoter level. This phenomenon has been demonstrated in several other milk proteins in the regulation of gene expression (Lechner, Welte, and Doppler 1997; Lechner *et al.* 1997; Stoecklin *et al.* 1997; Wyszomierski, Yeh, and Rosen 1999). It is also possible that Dex induces an autocrine secretion of prolactin, thereby initializing the signal transduction pathway leading to an activation of Stat5. The semi-quantitative results from this PCR amplification may therefore indicate a baseline activation of Stat5 in the cells not stimulated with prolactin, with further stimulation of binding induced by the addition of 10 nM human prolactin.

This experiment provides a biological application of the general method developed in the preparation of this dissertation, although it is restricted to analysis of known Stat5 response elements and does not involve cloning of new interaction sites.

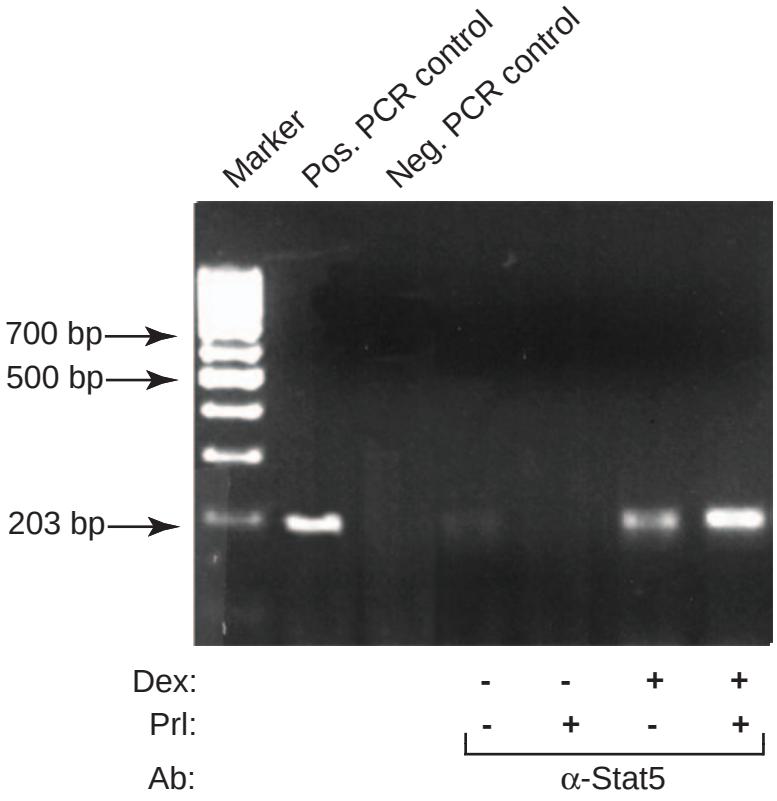


Figure 40. Stat5 is able to specifically associate with the promoter of α*S1-Casein* in T47D human breast cancer cells after glucocorticoid pre-treatment. Confluent cultures of cells were exposed to serum-free (- Dex) medium or Dexamethasone (+ Dex, 1μM) containing serum-free medium for 96 hours. Serum-free treated cultures contained identical volume of DMSO used as solvent for Dex. After pre-treatment parallel samples were treated with (+) or without (-) 10nM human Prl for 30 min and harvested as described. After glucocorticoid-induced differentiation Stat5 was able to bind the promoter for α*S1-Casein* and binding was further supplemented with Prl treatment, as determined by semi-quantitative PCR amplification. Non-specific IgG was used as a negative IP control and PCR was performed using primers designed to specifically amplify the promoters of the known Stat5-response element. Positive PCR control was performed using sonicated and purified Pre-IP sample for the template and negative PCR control was performed as a no-template reaction. Marker was 100 bp ladder.

The data clearly show that only after Dex-pretreatment can Stat5 bind to the promoter for *αS1-Casein*, indicating an alteration in the gene availability for the differentiation-related transcription factor Stat5.

β-Casein

β-Casein is the major casein constituent of human milk (Kunz and Lonnerdal 1990), comprising roughly 30% of the total protein content (Lonnerdal and Forsum 1985; Menon *et al.* 1992). Transcription and coactivators of the *β-Casein* gene have been thoroughly studied by a number of laboratories. It has been shown that a composite response region for the gene consists of binding sites for Stat5, CCAAT/Enhancer-Binding Protein-β (CEBP-β), and half sites for the GR (Wyszomierski and Rosen 2001). This response element is responsible for cooperative assimilation of signaling from prolactin, insulin, and hydrocortisone (or glucocorticoid).

In contrast to Stat5 interaction with the promoter of the *αS1-Casein* gene, the human *β-Casein* gene promoter was not available for binding of activated Stat5 under the conditions tested, regardless of whether or not Dex was present during the 96 h serum-free pretreatment period (Figure 41). The author concluded that the *β-Casein* promoter in T-47D cells is functionally shut down during the extended preincubation of T-47D cells in serum-free medium. As was previously shown in T-47D cells that were not cultured in the absence of serum for extended time, Stat5 activation led to specific binding to promoter of *β-Casein* (Figure 30).

These data provide interesting insight into the biological complexities of the cell and further illustrate that availability of promoters for transcription factor binding may be

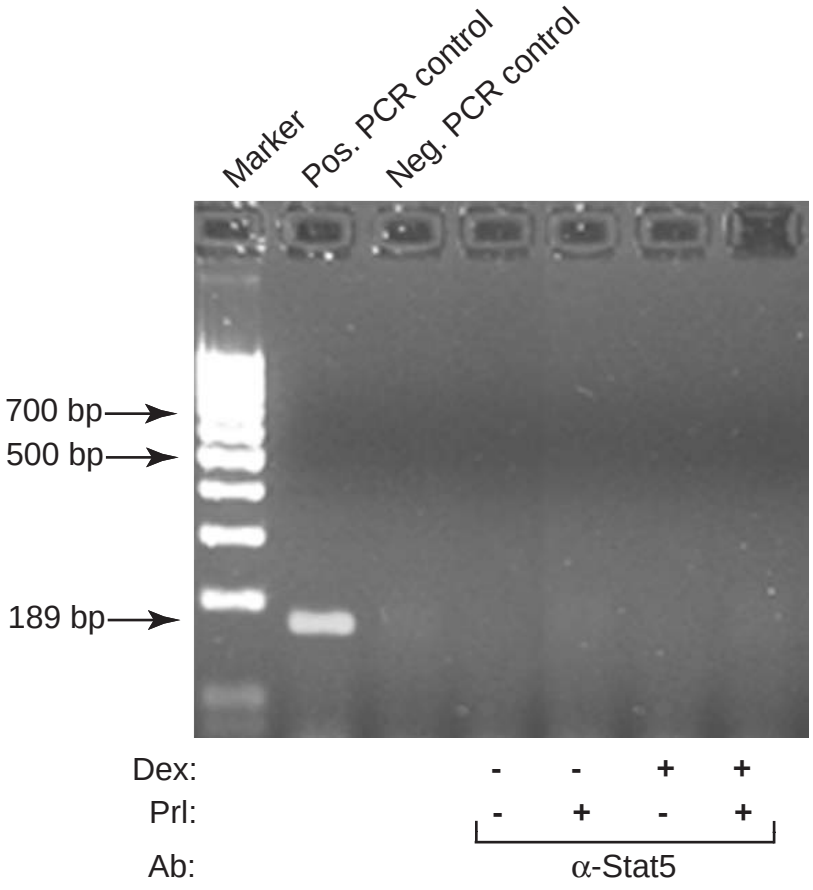


Figure 41. Prolactin activated Stat5 is not able to specifically associate with the promoter of β-*Casein* in T47D human breast cancer cells after glucocorticoid pre-treatment or starvation. Confluent cultures of cells were exposed to serum-free (- Dex) medium or Dexamethasone (+ Dex, 1μM) containing serum-free medium for 96 hours. Serum-free treated cultures contained identical volume of DMSO used as solvent for Dex. After pre-treatment parallel samples were treated with (+) or without (-) 10nM human Prl for 30 min and harvested as described. After starvation or glucocorticoid-induced differentiation and subsequent Prl treatment Stat5 was not able to bind the promoter for β-*Casein*. Non-specific IgG was used as a negative IP control and PCR was performed using primers designed to specifically amplify the promoters of the known Stat5-response element. Positive PCR control was performed using sonicated and purified Pre-IP sample for the template and negative PCR control was performed as a no-template reaction. Marker was 100 bp ladder.

highly regulated by serum factors and culture conditions. In humans, fluctuation in hormone levels from a number of factors, including various stimuli and circadian rhythms, modulate cell signaling and cellular responsiveness to external stimuli. Since it is well known that binding sites for GR exist within the response element for *β-Casein*, it is quite possible that persistent activation by Dex may inhibit the association of Stat5 with its respective binding site. Although speculative, such competitive inhibition of Stat5 binding may be related to the fact that in time of increased stress, milk production in females is decreased. In conclusion, in T-47D breast cancer cells Stat5 can bind thepromoter of *β-Casein* under standard culture conditions, however, serum starvation and/or Dex stimulation prohibits the association of Stat5 with this promoter *in vivo*.

Oncostatin M

Zarling, *et al* originally identified Oncostatin M (OSM) as a secreted glycoprotein found in conditioned medium from human leukemia cells that had been treated with phorbol 12-myristate 13-acetate (PMA), which caused the cells to differentiate into macrophage-like cells (Zarling *et al.* 1986). Subsequent analysis of the molecule revealed that it is a member of the Interleukin-6 (IL-6) family of cytokines, which regulate cell growth and differentiation in a large number of cell types. Primary and secondary sequence analysis revealed high similarity to leukemia inhibitor factor (LIF), granulocyte colony-stimulating factor (G-CSF), and IL-6; all of which have the ability to modulate differentiation in a diverse field of cell types (Rose and Bruce 1991). In other experimental models OSM has also been shown to act as a mitogen (Miles *et al.* 1992; Nair *et al.* 1992).

As eluded to, much of the work with OSM has been done in hematopoietic cell lines; however, OSM has the ability to inhibit the growth of A375 human melanoma cells, but not normal fibroblasts. Furthermore, recombinant OSM has been shown to inhibit growth in a number of cancerous cell lines originating from several different tissue types.

The critical nature of Stat5 involvement in OSM function and oncogenesis was also shown in a murine model (Schwaller *et al.* 1998; Schwaller *et al.* 2000). Stat5 deficient mice were infected with a retrovirus encoding a naturally occurring, constitutively active Stat5-tyrosine kinase fusion protein, TEL/JAK2. This experimental group of mice did not show any significant evidence of cancerous growth. However, when the same mice were given a bicistronic retrovirus containing TEL/JAK2 and Stat5a, the mice developed a rapidly fatal myelo- and lymphoproliferative disease. Furthermore, the same pathology could be induced by the addition of a constitutively active Stat5 mutant, eliminating the need for a constitutively active Stat5 tyrosine kinase, implying Stat5 is the driving force in oncogenesis. Importantly, further experiments implicated the Stat5 dependent OSM expression as a mediator of this pathology, thus connecting the function of OSM to the pathogenesis of the proliferative disease (Schwaller *et al.* 2000).

To specifically determine whether glucocorticoids modulated availability of the promoter of *OSM* for Stat5 binding, primers designed to amplify the promoter for *OSM* were used. Intriguingly, activated Stat5 bound to the promoter after 96 hours of serum deprivation and subsequent prolactin stimulation, but glucocorticoid pretreatment treatment completely eliminated the ability of Stat5 to associate with the promoter, with or without prolactin treatment (Figure 42). Presumably the Dex-induced differentiation

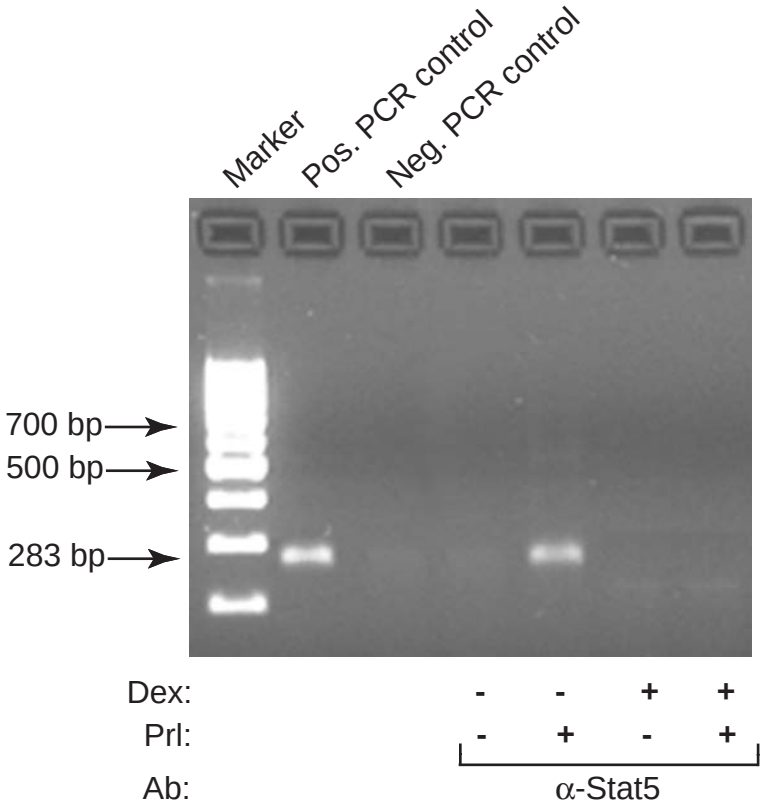


Figure 42. Prolactin activated Stat5 is able to specifically associate with the promoter of *OSM* in T47D human breast cancer cells, but not after glucocorticoid pre-treatment. Confluent cultures of cells were exposed to serum-free (- Dex) medium or Dexamethasone (+ Dex, 1μM) containing serum-free medium for 96 hours. Serum-free treated cultures contained identical volume of DMSO used as solvent for Dex. After pre-treatment parallel samples were treated with (+) or without (-) 10nM human Prl for 30 min and harvested as described. Activated Stat5 was able to associate with the promoter for *OSM*, but after glucocorticoid-induced differentiation and with or without Prl stimulation Stat5 was not able to bind the promoter for *OSM*. Non-specific IgG was used as a negative IP control and PCR was performed using primers designed to specifically amplify the promoters of the known Stat5-response element. Positive PCR control was performed using sonicated and purified Pre-IP sample for the template and negative PCR control was performed as a no-template reaction. Marker was 100 bp ladder.

alters the chromatin availability so that Stat5 is blocked from interaction with this specific response element. It should also be noted that in this case Dex treatment inhibits the ability of Stat5 to associate with its response element in *OSM*, whereas Dex treatment opened access to the *αS1-Casein* response element for binding by Stat5. Therefore, Dex-induced changes in chromatin access for Stat5 alters the pattern of interaction sites and does not simply cumulatively open up more and more sites. This observation that Dex will shut down the *OSM* promoter for Stat5 regulation may be important for modulating the response of the T-47D cells to prolactin.

Cyclin-Dependent Kinase Inhibitor 1A (CDKN1A)/ p21$^{WAF1/CIP1}$

Numerous studies performed on CDKN1A (p21$^{WAF1/CIP1}$) since it was first cloned in 1993 have to a large extent documented the function of this protein within the cell (el-Deiry *et al.* 1993; Harper *et al.* 1993). Generally, when activated, CDKN1A inhibits cell cycle progression by inhibiting the activation of cyclin kinases. CDKN1A is probably the main effector of p53 function in the cell, since its transcription is tightly controlled by p53 and mediates p53 suppression of tumor cell growth. Specifically, CDKN1A is critical for the G2 checkpoint of the cell cycle in human cells, and is activated by p53-induced activation in response to DNA damage (Bunz *et al.* 1998).

Stat5 relevance with respect to CDKN1A has been established in several cell lines of multiple origins, including hematopoietic and osteoblastic (Bellido *et al.* 1998; Matsumura *et al.* 1997). Because Stat5 is a critical factor for terminal differentiation of breast epithelial cells, it is possible that Stat5 upregulates CDKN1A as part of growth suppression needed for differentiation to take place. It was therefore of particular

interest to examine whether glucocorticoid pretreatment of T-47D cells would affect the access of Stat5 to the *CDKN1A* promoter. As illustrated in Figure 43, accessibility to the Stat5 response element was markedly Dex-dependent and only after prolactin stimulation could Stat5 bind to the promoter. This result can be interpreted as follows: after Dex-induced differentiation there is an alteration in chromatin access that allows activated Stat5 to bind to the *CDKN1A* promoter. If transferable to normal breast epithelial cells, this concept would be consistent with an inhibition of cell cycle given that the cells are terminally differentiated. The addition of prolactin initiates signal transductionthat activates Stat5 to function as the regulator of functional mammary epithelial cells, as has been shown by several methods.

CIS1

Cytokine-inducible SH2 protein 1 (CIS1), has several alternate names and symbols in *Homo sapiens* including Stat-induced Stat inhibitor I (SSI-1), Jak binding protein (JAB), TEC-interacting protein 3 (TIP3), and suppressor of cytokine signaling 1 (SOCS1) according to the official gene characterization at the National Center for Biotechnology Information (NCBI) at the National Institues of Health (NIH), Bethesda, MD (http://www.ncbi.nlm.nih.gov/LocusLink and http://www.ncbi.nlm.nih.gov/OMIM). CIS1 is a well-documented auto-regulated inhibitor of cytokine signaling through the Jak-Stat pathway (Matsumoto *et al.* 1997). As previously mentioned, it is absolutely necessary to maintain tight control of signal transduction pathways, both for rapid induction and cessation of signaling. The protein CIS1 was isolated independently for its

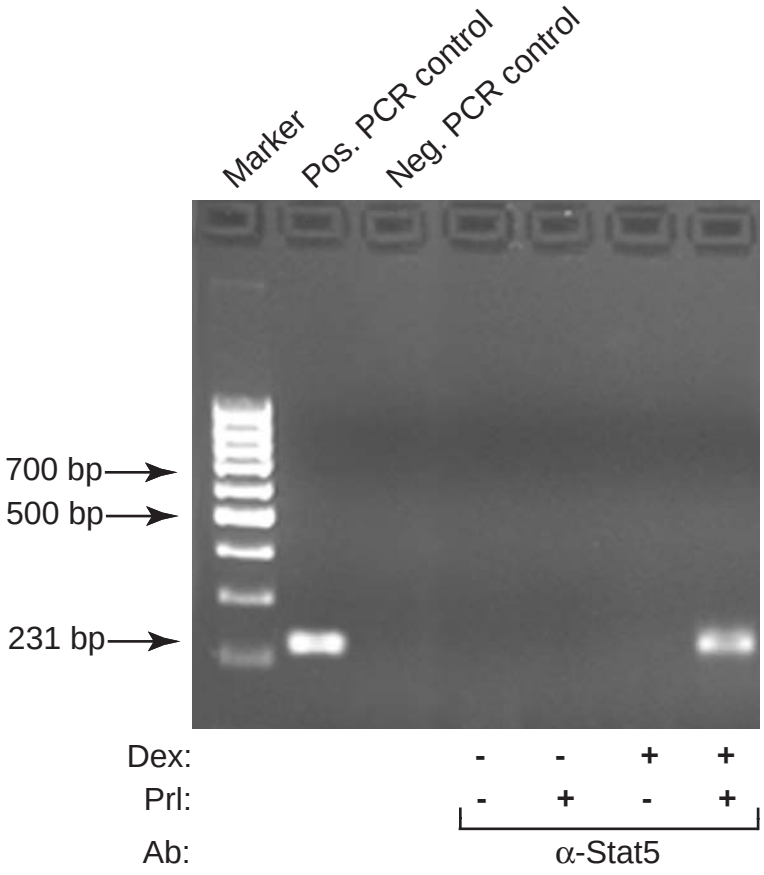


Figure 43. Prolactin activated Stat5 is able to specifically associate with the promoter of *CDKN1A* (*p21*$^{WAF1/CIP1}$) in T47D human breast cancer cells only after glucocorticoid pre-treatment. Confluent cultures of cells were exposed to serum-free (- Dex) medium or Dexamethasone (+ Dex, 1μM) containing serum-free medium for 96 hours. Serum-free treated cultures contained identical volume of DMSO used as solvent for Dex. After pre-treatment parallel samples were treated with (+) or without (-) 10nM human Prl for 30 min and harvested as described. Only after glucocorticoid-induced differentiation and Prl stimulation was Stat5 able to bind the promoter for *CDKN1A.* Non-specific IgG was used as a negative IP control and PCR was performed using primers designed to specifically amplify the promoters of the known Stat5-response element. Positive PCR control was performed using sonicated and purified Pre-IP sample for the template and negative PCR control was performed as a no-template reaction. Marker was 100 bp ladder.

ability to interact with Jak2 in a 2-hybrid screen and as a molecule that inhibited IL-6-induced differentiation of murine monocytic M1 cells (Starr *et al.* 1997).

Subsequent work has shown CIS1 to be required for *in vivo* regulation of multiple cell types and is absolutely critical for normal postnatal growth and survival in mice (Starr *et al.* 1998). Furthermore, *CIS1* has been identified as a candidate tumor suppressor gene from work done in hepatocellular carcinoma patient samples (Yoshikawa *et al.* 2001). Specifically, the gene locus was methylated (silenced) in a majority of patients, leading to constitutive activation of the Jak-Stat pathway (Zardo *et al.* 2002).

Experiments in T-47D cells to determine Stat5 responsiveness to prolactin-induced activation showed a definitive association with the promoter for *CIS1*. Only after Dex treatment and prolactin stimulation could Stat5 bind the promoter (Figure 44). In parallel cell cultures that were serum starved in the absence Dex for 96 hours and then stimulated with prolactin, Stat5 could not bind the promoter. These data also revealed a dependence of the *CIS1* promoter on Dex for access by activated Stat5. It is important to note that in the negative immunoprecipitation control samples, no detectable product could be seen, attesting to the specificity for the association of Stat5 with the *CIS1* promoter.

It will be interesting to determine whether loss of Stat5-inducible *CIS1* regulation during serum-starvation of T-47D cells is reflected in a hypersensitization of the cells to Stat5 activation. For instance, will prolactin-induced Stat5 activation last longer in T-47D cells that are serum-starved in the absence of Dex, compared to the duration of signal in Dex-treated cells where Stat5 has access to the *CIS1* promoter? Further work may shed new light on steroid control of the *CIS1* promoter in breast cancer.

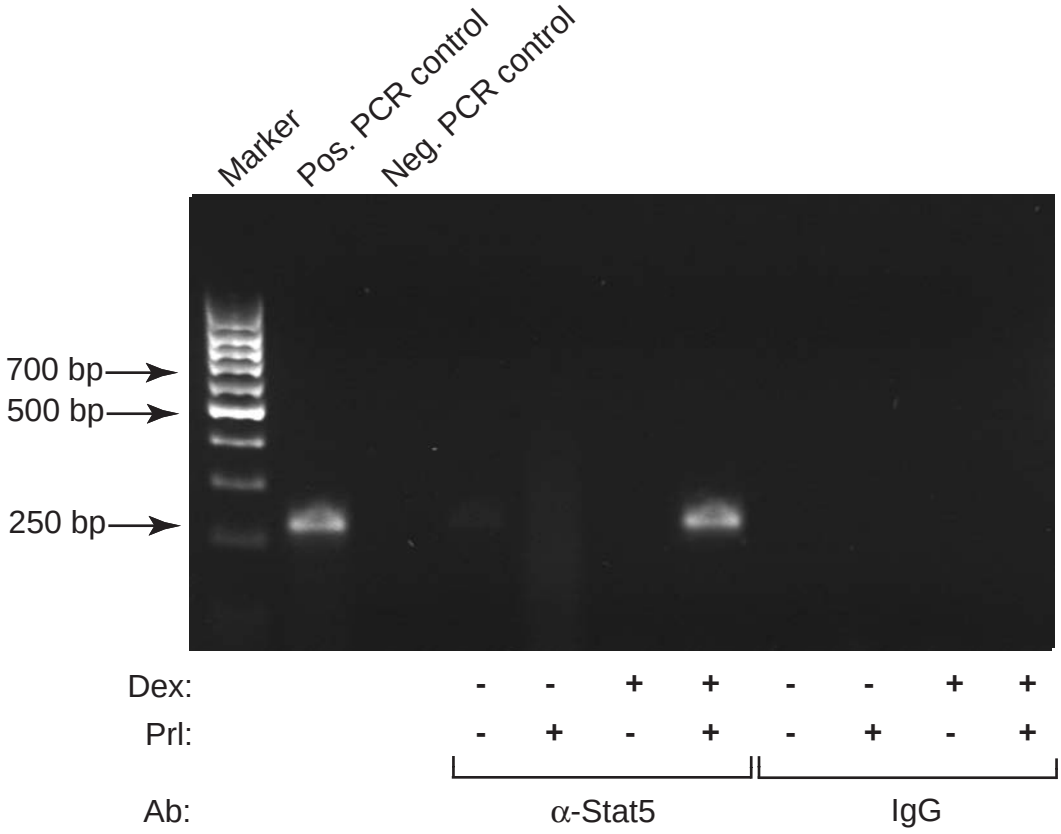


Figure 44. Prolactin activated Stat5 is able to specifically associate with the promoter of *CIS1* in T47D human breast cancer cells only after glucocorticoid pre-treatment. Confluent cultures of cells were exposed to serum-free (- Dex) medium or Dexamethasone (+ Dex, 1μM) containing serum-free medium for 96 hours. Serum-free treated cultures contained identical volume of DMSO used as solvent for Dex. After pre-treatment parallel samples were treated with (+) or without (-) 10nM human Prl for 30 min and harvested as described. Only after glucocorticoid-induced differentiation and Prl stimulation was Stat5 able to bind the promoter for *CIS1*. Non-specific IgG was used as a negative IP control and PCR was performed using primers designed to specifically amplify the promoters of the known Stat5-response element. Positive PCR control was performed using sonicated and purified Pre-IP sample for the template and negative PCR control was performed as a no-template reaction. Marker was 100 bp ladder.

PIM1

PIM1 is a serine/threonine kinase and has been established as a protooncogene in humans. Analysis of gene expression has shown a preponderance of expression in B-lymphoid and myeloid cell lines (Domen *et al.* 1987; Meeker *et al.* 1987) and in sites of fetal hematopoiesis (Amson *et al.* 1989), however, expression has also been shown to be significantly upregulated in prostate cancer (Dhanasekaran *et al.* 2001). However, PIM1 gene deletion and transgenic overexpression of the protein in mice have not directly linked the protein with an oncogenic phenotype and only showed differences in erythrocyte size (Laird *et al.* 1993).

Experiments in T-47D human breast cancer cells showed definitive, inducible Stat5 interaction with the promoter for *PIM1*. Activation of Stat5 by prolactin administration brought about Stat5 binding to the promoter as seen in Figure 45. While Dex pretreatment of the cells moderately increased the overall association of Stat5 with the promoter when stimulated with prolactin, Stat5 was able to bind the promoter regardless of whether the cells were pretreated with Dex or not. Interestingly, when the cells were pretreated with glucocorticoids, Stat5 was able to bind to the promoter at a baseline level, even without exogenous prolactin stimulation. A similar phenomenon was observed for the *αS1-Casein* promoter, which also showed this same low-level, intrinsic association of Stat5 when pretreated for 96 hours with Dex.

While the targets for the serine/threonine kinase activity of PIM1 have not been established, it is interesting to note that in BCR/ABL transformed cells, Stat5 activation is required (Nieborowska-Skorska *et al.* 2002). One role of Stat5 in BCR/ABL

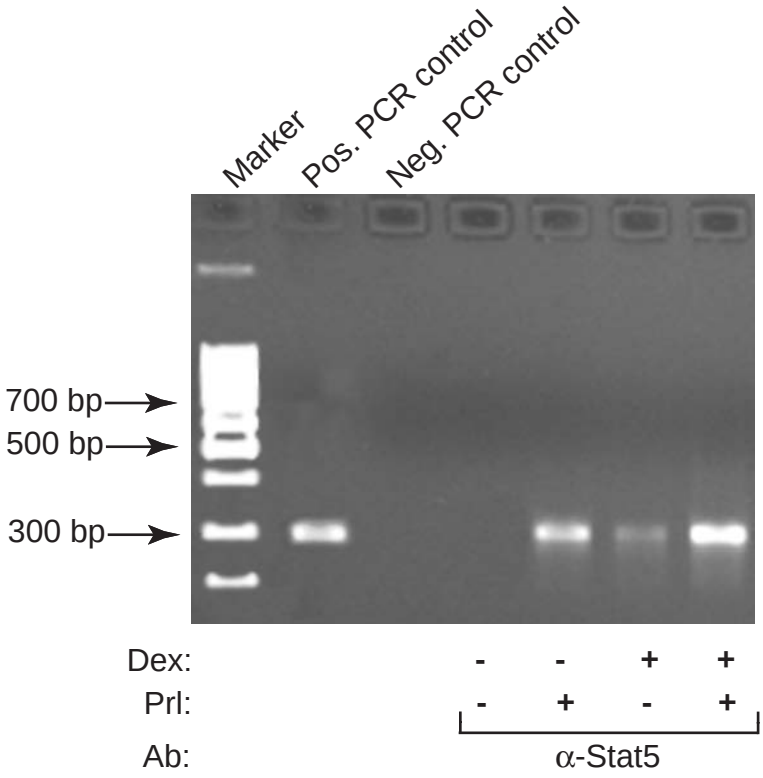


Figure 45. Prolactin activated Stat5 is able to specifically associate with the promoter of *PIM1* in T47D human breast cancer cells and is further induced after glucocorticoid pre-treatment. Confluent cultures of cells were exposed to serum-free (- Dex) medium or Dexamethasone (+ Dex, 1μM) containing serum-free medium for 96 hours. Serum-free treated cultures contained identical volume of DMSO used as solvent for Dex. After pre-treatment parallel samples were treated with (+) or without (-) 10nM human Prl for 30 min and harvested as described. Only after Prl stimulation could Stat5 associate with the promoter for *PIM1*, however after glucocorticoid-induced differentiation Stat5 was able to bind the promoter for *PIM1* and was further induced with Prl stimulation. Non-specific IgG was used as a negative IP control and PCR was performed using primers designed to specifically amplify the promoters of the known Stat5-response element. Positive PCR control was performed using sonicated and purified Pre-IP sample for the template and negative PCR control was performed as a no-template reaction. Marker was 100 bp ladder.

transformed cells may be the upregulation of PIM1 expression, possibly contributing to the protection from apoptosis in these cells.

Additional Stat5-Regulated Genes

In addition to the previously mentioned genes, primer sets were also designed to flank the known Stat5 binding site in identified Stat5-responsive genes. The results from the PCR amplification of Stat5-mediated pull-down of Stat5-chromatin interaction sites are shown here. The genes include: *α2-Macroglobulin* (Figure 46 – Dex- and prolactin induced binding of Stat5), *Estrogen Receptor α* (Figure 47 – no Stat5 binding), *PRLR* (Figure 48 - no Stat5 binding), and *IL-2Rα* (Figure 49 – no Stat5 binding).

Discussion

The goal of these experiments was to use the new technology developed in this dissertation to determine whether glucocorticoids affected the pattern of gene promoter association by activated Stat5 using the human T-47D breast cancer cell as a model system. This question would apply the chromatin-interaction analysis to a biologically relevant setting. The T-47D cell line is a relatively well-differentiated human breast cancer cell line and has been shown to be responsive to glucocorticoid treatment. Here, cells were either starved for 96 hours in serum-free medium or treated with Dex and then analyzed for their responsiveness to prolactin stimulation with respect to Stat5 activation.

As illustrated from these experiments, pro-differentiation treatment markedly altered the pattern of available Stat5 responsive sites, presumably a reflection of a different gene expression profile. What was especially interesting, however, was the

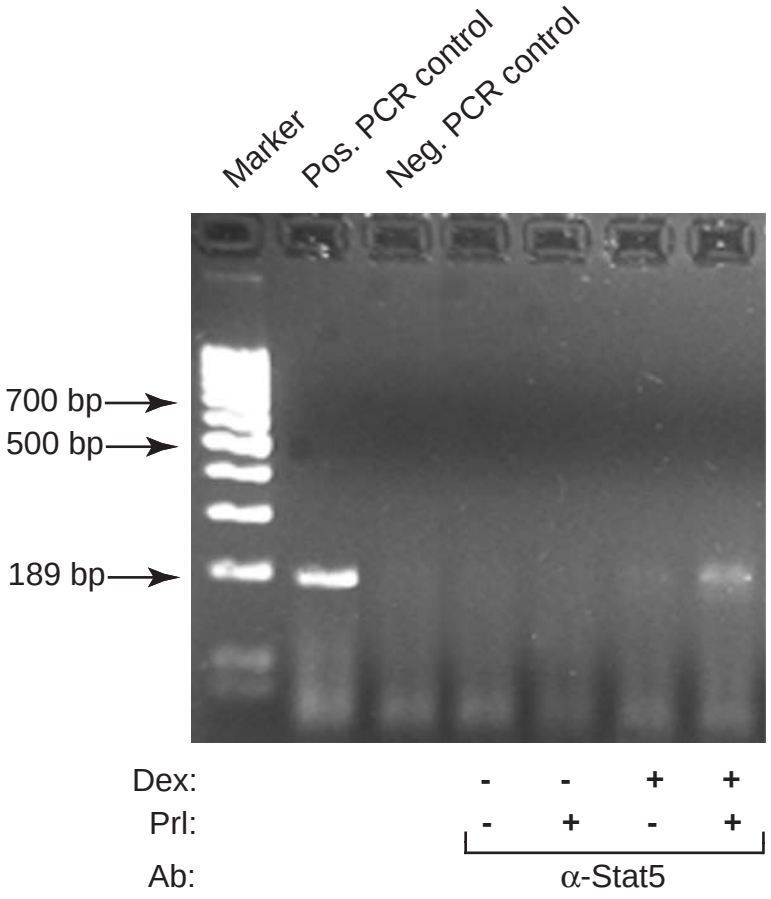


Figure 46. Prolactin activated Stat5 is able to specifically associate with the promoter of α*2-Macroglobulin* in T47D human breast cancer cells only after glucocorticoid pre-treatment. Confluent cultures of cells were exposed to serum-free (- Dex) medium or Dexamethasone (+ Dex, 1μM) containing serum-free medium for 96 hours. Serum-free treated cultures contained identical volume of DMSO used as solvent for Dex. After pre-treatment parallel samples were treated with (+) or without (-) 10nM human Prl for 30 min and harvested as described. Only after glucocorticoid-induced differentiation and Prl stimulation was Stat5 able to bind the promoter for α*2-Macroglobulin*. Non-specific IgG was used as a negative IP control and PCR was performed using primers designed to specifically amplify the promoters of the known Stat5-response element. Positive PCR control was performed using sonicated and purified Pre-IP sample for the template and negative PCR control was performed as a no-template reaction. Marker was 100 bp ladder.

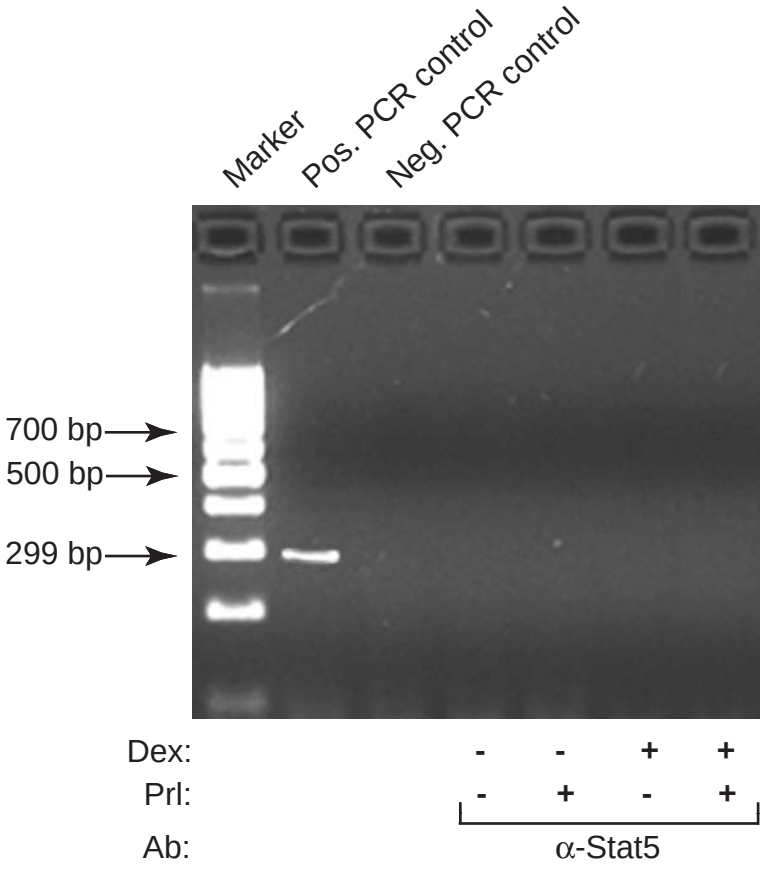


Figure 47. Prolactin activated Stat5 is not able to associate with the promoter of *Estrogen Receptor* α in T47D human breast cancer cells after glucocorticoid pre-treatment or starvation. Confluent cultures of cells were exposed to serum-free (- Dex) medium or Dexamethasone (+ Dex, 1μM) containing serum-free medium for 96 hours. Serum-free treated cultures contained identical volume of DMSO used as solvent for Dex. After pre-treatment parallel samples were treated with (+) or without (-) 10nM human Prl for 30 min and harvested as described. Regardless of glucocorticoid-induced differentiation and with or without Prl stimulation Stat5 was not able to bind the promoter for *ER*α. Non-specific IgG was used as a negative IP control and PCR was performed using primers designed to specifically amplify the promoters of the known Stat5-response element. Positive PCR control was performed using sonicated and purified Pre-IP sample for the template and negative PCR control was performed as a no-template reaction. Marker was 100 bp ladder.

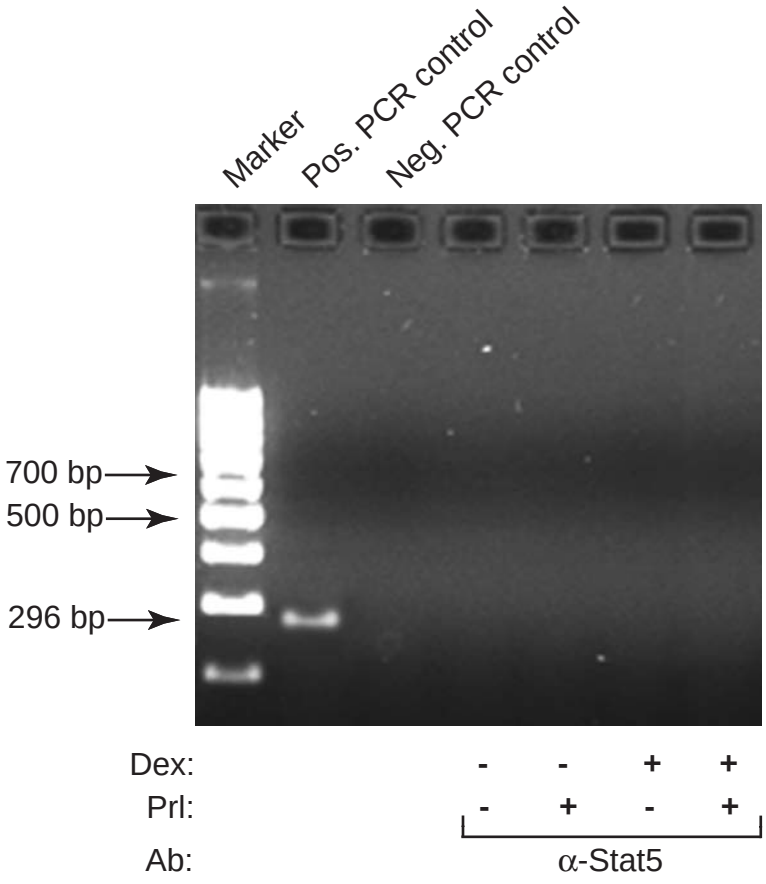


Figure 48. Prolactin activated Stat5 is not able to associate with the promoter of *PRLR* in T47D human breast cancer cells after glucocorticoid pre-treatment or starvation. Confluent cultures of cells were exposed to serum-free (- Dex) medium or Dexamethasone (+ Dex, 1μM) containing serum-free medium for 96 hours. Serum-free treated cultures contained identical volume of DMSO used as solvent for Dex. After pre-treatment parallel samples were treated with (+) or without (-) 10nM human Prl for 30 min and harvested as described. After starvation or glucocorticoid-induced differentiation and subsequent Prl treatment Stat5 was not able to bind the promoter for *PRLR*. Non-specific IgG was used as a negative IP control and PCR was performed using primers designed to specifically amplify the promoters of the known Stat5-response element. Positive PCR control was performed using sonicated and purified Pre-IP sample for the template and negative PCR control was performed as a no-template reaction. Marker was 100 bp ladder.

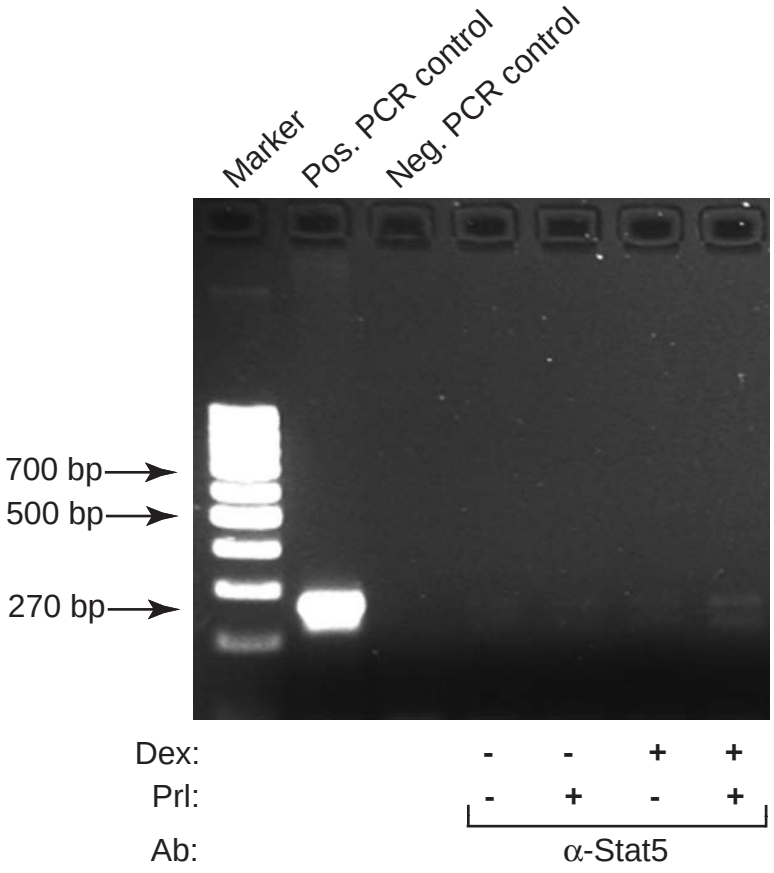


Figure 49. Prolactin activated Stat5 is not able to associate with the promoter of *IL-2R*α in T47D human breast cancer cells after glucocorticoid pre-treatment or starvation. Confluent cultures of cells were exposed to serum-free (- Dex) medium or Dexamethasone (+ Dex, 1μM) containing serum-free medium for 96 hours. Serum-free treated cultures contained identical volume of DMSO used as solvent for Dex. After pre-treatment parallel samples were treated with (+) or without (-) 10nM human Prl for 30 min and harvested as described. Regardless of glucocorticoid-induced differentiation and with or without Prl stimulation Stat5 was not able to bind the promoter for *IL2R*α. Non-specific IgG was used as a negative IP control and PCR was performed using primers designed to specifically amplify the promoters of the known Stat5-response element. Positive PCR control was performed using sonicated and purified Pre-IP sample for the template and negative PCR control was performed as a no-template reaction. Marker was 100 bp ladder.

demonstration that access to regulatory chromatin sites may be either opened or closed on an individual basis in response to the same treatment. The use of this novel methodology provided a powerful and unique means to evaluate the status of Stat5 involvement in cellular processes by directly revealing a physical association of Stat5 with a specific response element. Table 6 summarizes the effect of glucocorticoid treatment on accessibility of a panel of Stat5 interaction sites.

Additional experiments will further explain the role of Stat5 in these very important processes of cell differentiation and development. This more complete understanding of biological processes will aid in the advancement of more specific and effective treatments that can be individually tailored to the characteristics of each malignancy.

Materials and Methods

Glucocorticoid Induced Mammary Cell Differentiation

Cultures of T-47D cells were treated with the synthetic glucocorticoid Dexamethasone (Dex) to induce a phenotypic differentiation. After growing to confluence in standard growth medium, the culture flask medium was replaced with either serum free medium or serum free medium containing 1 μM Dex. Stock solutions of 10 mM Dex were prepared by dissolving the appropriate quantity of Dex in dimethyl sulfoxide (DMSO). In cultures treated with serum free medium without Dex, an identical quantity of DMSO was added as a control.

Cells were kept in a tissue culture incubator at 37°C with 5% CO_2 for 96 hours to induce differentiation in the T-47D human breast cancer cells. After treatment each

Table 6. Summary of Cell Differentiation-Dependent Changes in Accessibility of Specific Genomic Response Elements to Transcription Factor Stat5

	- Dex / - Prl	*- Dex / + Prl*	*+ Dex / - Prl*	*+ Dex / + Prl*
αS1-Casein	-	-	+	+++
α2-Macroglobulin	-	-	-	+
β-Casein	-	-	-	-
CIS1	-	-	-	+++
ERα	-	-	-	-
IL-2Rα	-	-	-	-
OSM	-	++	-	-
CDKN1A	-	-	-	++
PIM1	-	++	+	+++
PRLR	-	-	-	-

group (+ Dex and – Dex) were divided in half and were treated with or without 10 nM human Prl for 30 minutes as described elsewhere. The samples then were prepared as described for the purification of Stat5-bound response elements.

PCR Primer Design of Known Stat5 Binding Elements

After the final step in the recovery of the elements, the samples were subjected to PCR amplification with primer sets designed to specifically flank known Stat5 binding sites in the promoters of Stat5 responsive genes. Although not identical, the primers were designed with highly similar melting and annealing temperatures as well as comparable product sizes, so consistent PCR cycling parameters could be used. The oligonucleotides were synthesized by the Biomedical Instrumentation Center (BIC) at USUHS or by Invitrogen (Carlsbad, CA). The primer sets were as follows: *αS1-Casein* forward 5' CCA AAA CAC ATA GGA CAG TTG G 3', reverse 5' TGG GGA CAA AAT AGG GTC TT 3'; *α2-Macroglobulin* forward 5' TTT AGC CCT CCA GGG ATT CT 3', reverse 5' CAA TCC ATC TGG TCC CAA AC 3'; *β-Casein* forward 5' GGA GAA ACA GTT TGC CTC ACA 3', reverse 5' CCT AGT GGG GCC TTG AGA TT 3'; *BCL-X_L* forward 5' TAC AAA AGA TCT TCC GGG GG 3', reverse 5' CCC CTC CAG GTA CCA GAA CT 3'; *CIS-1* forward 5' CTA TTG GCC CTC CCC GAC 3', reverse 5' AGC TGC TGC CTA ATC CTT TG 3'; *Cyclin D1* forward 5' GAA ACT TGC ACA GGG GTT GT 3', reverse 5' ATT TAG GGG GTG AGG TGG AG 3'; *ERα* forward 5' TGC TGT TCT CGT GGT AAT GAA 3', reverse 5' CCC ACA GCA TGG ACT TCT CT 3'; *IL-2Rα* forward 5' CAT TTC AAT TGC TCT TCT TAC CA 3', reverse 5' GGA ACA AGT TCA AGA AAG GAA CA 3'; *OSM* forward 5' AAG TCC

CTC CTG CCC ATC 3', reverse 5' CGA TTG GCC AAC ACC TCA T 3'; *CDKN1A (P21$^{Waf1/Cip1}$)* forward 5' CTC TCC AAT TCC CTC CTT CC 3', reverse 5' CTG CAA TTT CCA GAA AAG CC 3'; *PIM1* forward 5' CAC CCT CCC ACC CTA GTT TT 3', reverse 5' ACA TGA GTC ACG GAG GGA GT 3'; *PrlR* forward 5' ATG AGG ACT TGC TGG TGG AG 3', reverse 5' ATA GGG GAT TTT GCC TTC CA 3'.

The PCR was performed under standard conditions in either a 25 µl or 50 µl reaction volume. Most of the amplifications were performed using Qiagen (Valencia, CA) *Taq* PCR master mix set that contained the appropriate reaction buffer, magnesium concentration, nucleotides, and *Taq* polymerase in a 2X stock concentration. The respective template and primers were added for each reaction. Some experiments were performed with *Taq* polymerase (Fermentas Hanover, MA) and the relevant components as listed above. No difference in results of PCR products could be seen from either method of amplification. The sample was initially heated to 94°C for 2 minutes, then cycled 36 times at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 20 seconds. Each set of samples contained a positive and negative PCR control, in addition to the experimental samples. The positive control was either purified genomic DNA or a portion of the sonicated DNA removed as a pre-immunoprecipitation sample, which was subsequently purified by phenol:chloroform extraction and ethanol precipitation. Regardless of the method, both were isolated from the same T-47D cell line and showed no difference in the amplification results. The negative PCR control was performed by using a no-template reaction however contained the same reaction components.

After amplification the products were combined with a 6X DNA loading buffer (Fermentas, Hanover, MA) and added to a 2% TBE-agarose gel and run for

approximately 1 hour at 120 volts. Upon completion, the gel was visualized under ultra-violet light and was documented using an Eagle Eye II documentation system (Stratagene, La Jolla, CA) or more recently a Quantity One Gel Doc system (Bio-Rad, Hercules, CA).

CONCLUDING REMARKS

The work presented in this dissertation centers on the development of new strategies to identify target genes for the transcription factor Stat5. The immediate rationale and justification for developing genome-wide technologies to identify Stat5 target genes lies in novel observations in the mentor's laboratory, which suggest that Stat5 serves as a molecular guardian against metastatic invasion of human breast cancer. Supporting this notion, additional evidence of a growth-suppressive, prodifferentiation role for the Jak2-Stat5 pathway in breast epithelial cells was presented in the first part of this dissertation. However, because Stat5 is important in biological processes other than breast epithelial growth and differentiation, including hematopoiesis, immune system function, ovarian steroid production, prostate cancer cell survival, and somatic growth, improved technologies to identify Stat5-target genes may also facilitate progress in many areas of physiology and pathology.

Two independent strategies for genome-wide identification of new Stat5 target genes were established. First, a cloning and validation procedure was established for effective identification of Stat5-bound chromatin interaction sites. This methodology was applied to human breast cancer cells and a validation procedure to verify Stat5-binding to the captured fragment was developed. Proof-of-principle for this method has been presented, and three out of three candidate target sequences isolated were fully validated as inducible chromatin interaction sites for Stat5. Further work will now use this protocol and test the additional 30 candidate Stat5-binding fragments isolated in the first round of cloning, as well as continue the cloning of additional chromatin fragments sites with the long-term goal of generating a genome-wide map of Stat5 interaction sites.

This ambitious effort is planned to become a part of the second phase of the human genome project that is just being initiated, Encyclopedia of DNA Elements or ENCODE, which aims to map all functional elements of the human genome.

A second strategy to identify Stat5 target genes was devised, which takes advantage of adenoviral gene delivery of dominant-negative Stat5 to identify transcripts that are rapidly induced by Stat5 among a panel of prolactin-induced transcripts. This strategy involved acute activation of Stat5 in breast cancer cells, using prolactin as a trigger in the presence or absence of a dominant-negative, transcriptionally blocking mutant of Stat5. By isolating RNA from cells under the different activation conditions, transcripts that were induced by prolactin in the presence of wild type Stat5, but not in the presence of dominant-negative Stat5, represented candidate Stat5 target genes. In other words, this strategy could, with high level of confidence, distinguish between Stat5-mediated and non-Stat5-mediated, prolactin-inducible genes. Successful application and proof-of-principle of this approach was achieved, paving the way for broader and more extensive analyses. By combining this mRNA-based method with the chromatin-interaction based method, future plans involve rapid identification of the Stat5-interaction sites in the promoters of these newly discovered Stat5-inducible genes.

The chromatin-interaction based technology was also applied in this dissertation research to specifically determine whether glucocorticoids modify chromatin access of Stat5 in breast cancer cells. Glucocorticoids are known to cooperate with prolactin to enhance the terminal differentiation of breast epithelial cells and the author hypothesized that glucocorticoids would change the pattern of available chromatin binding sites for Stat5. This work verified the hypothesis and led to the conclusive observation that the

promoters of some Stat5 regulated genes became accessible for Stat5 binding, others became inaccessible, while yet others remained relatively unaltered.

As one useful future application of determining interaction of a transcription factor with a particular promoter, the author and Dr. Rui predict that certain key interaction sites will yield important information to predict tumor behavior. For instance, Stat5 binding to the promoter of the growth-inhibitory $p21^{WAF1/CIP1}$ (*CDKN1A*) gene in a breast cancer specimen may signify favorable prognosis or predict responsiveness to certain therapeutic alternatives. Such new high-resolution tumor markers may add valuable and refined information beyond what simple immunohistochemical determination of transcription factor status does and could lead to a new diagnostic paradigm in molecular pathology.

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